Alzheimer's disease and neuronal survival

Laura Mussalo
M. Sc. Thesis
Master of Science in Pharmacy degree
University of Eastern Finland
School of Pharmacy
Pharmacology
June 2013

UNIVERSITY OF EASTERN FINLAND, Faculty of Health Sciences
School of pharmacy
Master of science in Pharmacy degree
Pharmacology
LAURA MUSSALO: Alzheimer’s disease and neuronal survival
Master's thesis, 68 p.
Supervisors: Ph.D. José Ramon Bayascas and Ph.D. Anne Lecklin
June 2013

Keywords: Alzheimer's disease, β- amyloid, amyloid cascade hypothesis, AKT, BAD

Alzheimer’s disease (AD) is a progressive neurodegenerative disease which prevalence is increasing as the population ages. It is the most common cause of dementia on elderly and it covers over 60% of all dementias. It is estimated that about 33% of over the age of 85 has it. There are two forms of the disease, familial and sporadic where familial is rare and sporadic form is generalizing alongside the aging population.

Behind the pathogenesis of AD is altered processing and deposition of two proteins: β- amyloid and tau. These proteins accumulate to the brain and cause typical pathological changes: amyloid plaques and neurofibrillary tangles that cause eventually death of neurons. This leads to progressive decline of cognitive function. At this moment, there is no curative or disease- modifying treatment. Only symptomatic therapies are available to alleviate some manifestations of the disease.

Cholinesterase inhibitors are standard first- line drugs used to treat AD. Especially dual therapy with NMDA- receptor antagonist memantine show considerable improvement in cognitive functions and activities of daily living. New treatments have been developed vigorously over two decades and a lot of time and effort has put on targeting β- amyloid. Different compounds affecting its formation, aggregation and degradation have developed yet without clinical breakthrough. Effect of sex hormones on AD also intrigues researchers and selective sex hormone receptor modulators are under investigations.

AKT also known as protein kinase B is a serine/ threonine kinase that is activated downstream mainly by insulin. It regulates many important events such as growth, metabolism, proliferation and survival. AKT belongs to the class of AGC- kinases. One of its substrates is pro- apoptotic BAD (BCL- 2 associated death protein) which is inhibited by phosphorylation by AKT.

Aim of the study was to investigate whether impaired AKT activation affect on phosphorylation of BAD. This was performed by developing a protocol that enables best conditions to detect BAD in its phosphorylated state. When conditions were balanced, experiments were performed with specific PDK1 K465E- neurons developed in the laboratory of Dario R. Alessi. In these neurons AKT activation is known to be disrupted.

The results of the present study indicated that phosphorylation of BAD is impaired in PDK1 K465E- neurons. However compared to the earlier findings with other AKT substrates results were deviating. Further investigation is needed with PDK1 K465E- neurons to demonstrate whether BAD phosphorylation is affected or not.

ITÄ-SUOMEN YLIOPISTO, terveystieteiden tiedekunta
Farmasian laitos
Proviisorin koulutusohjelma
Farmakologia
MUSSALO LAURA: Alzheimerin tauti ja neuraalinen selviytyminen
Pro gradu- tutkielma, 68 s.
Ohjaajat: FT José Ramon Bayascas, FT Anne Lecklin

Avainsanat: Alzheimerin tauti, amyloidikaskadihypoteesi, AKT, BAD

Alzheimerin tauti (AT) on progressiivinen neurodegeneratiivinen sairaus jonka esiintyvyys kasvaa väestön ikääntyessä. Se on dementian yleisin syy iäkkäillä, kattaen yli 60% kaikista dementioista. Arviolta 33% yli 85- vuotiaista sairastaa Alzheimerin tautia. Taudista kaksi muotoa: familiaalinen eli perinnöllinen sekä sporadinen eli satunnainen. Familiaalinen muoto on harvinainen kun taas sporadinen muoto on yleistymässä ikääntyvän väestön rinnalla.

Alzheimerin taudin patogeneesin aiheuttaa β- amyloidin ja tau- proteiinin häiriintynyt prosessointi ja kertyminen aivoihin. Tämä aiheuttaa tyypilliset patologiset muutokset: amyloidiplakit ja neurofibrillikimput jotka aiheuttavat lopulta hermosolujen kuoleman. Tämä johtaa etenevään kognition heikkenemiseen. Parantavaa tai merkittävästi taudin kulkuun vaikuttavaa hoitoa ei vielä ole kehitetty. Olemassaolevat lääkitykset ainoastaan helpottavat joitain tautiin liittyviä oireita.

Antikoliiniesteraasit ovat Alzheimerin taudin hoidon peruslääkkeitä. Erityisesti yhdistettynä NMDA- reseptori antagonistiin memantiiniin, lääkkeillä on selkeä positiivinen vaikutus kognitioon ja toimintakykyyn. Uusia hoitoja on kehitetty jo yli kahden vuosikymmenen ajan mutta toistaiseksi ilman läpimurtoja. β- amyloidien muodostumiseen, aggregaatioon ja kertymiseen vaikuttavien lääkkeiden kehittäminen on yksi keskeisistä kohteista. Sukupuolihormonien vaikutukset ovat myös mielenkiinnon kohteena ja kehityksen alla on selektiivisiä sukupuolihormonireseptorien muuntelijoita.

AKT joka tunnetaan myös nimellä proteiini kinaasi B on insuliinivälitteinen seriini/ treoniinikinaasi. Se säätelee monia tärkeitä tapahtumia, kuten solujen kasvua, metaboliaa ja selviytymistä. AKT kuuluu AGC- kinaasien luokkaan. Pro-apoptoottinen BAD (BCL- 2 associated death protein)- kinaasi on yksi AKT:n substraateista. AKT aiheuttaa BAD:in fosforyloitumisen, ja tämä estää sen toimintaa.

Tutkimuksen tavoitteena oli tutkia onko häiriintyneellä AKT:n aktivaatiolla vaikutusta BAD:in fosforylaatioon. Tätä varten määriteltiin parhaat olosuhteet joissa BAD voidaan havaita sen fosforyloituneessa muodossa. Tämän jälkeen kokeita suoritettiin käyttäen Dario R. Alessin laboratoriossa kehitettyjä PDK1 K465E- neuroneita, joissa AKT aktivaation tiedetään olevan häiriintynyt.

Suoritetun tutkimuksen perusteella näyttäisi että BAD- kinaasin fosforylaatio on alentunut PDK1 K465E- neuroneissa. Kuitenkin, verrattuna aiempiin tuloksiin muiden substraattien osalta, tulokset ovat poikkeava. PDK1 K465E- neuroneilla täytyy suorittaa lisätutkimuksia jotta yhteys BAD- kinaasin fosforylatioon voidaan vahvistaa.

Preface

Writing this thesis has been a long process that started in 2012 in Barcelona by working in the group of José Ramón Bayascas. I want to dedicate special thanks to him for taking me into his group and providing this opportunity to study/ work abroad. I also want to thank Lluis, Xiangyu, Victoria, Maria, Elisenda, Nabil and especially Tinatin Zurashvili who guided me patiently through my work and always supported me. Thanks to Anne Lecklin who encouraged me to do this work and guided me through whole process. I want also dedicate warm thanks to my friends and family who pushed me forward non- stop. Last but not least I want to say to my husband Akseli Mussalo who married me in the middle of this process; without your support this would not ever happened.

Laura Mussalo
16.6.2013 Kuopio

Abbreviations

Aβ	β- amyloid
ACE	Angiotensin- converting enzyme
AD	Alzheimer's disease
AICD	Amyloid intracellular domain
AKT	Protein kinase B (see PKB)
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
A1	BCL- 2- related gene A1
BACE1	β- site APP- cleaving enzyme 1
BAD	BCL- 2 antagonist of cell death/ BCL- 2- associated death protein
BAX	BCL- 2- associated x protein
BAK	BCL- 2 antagonist killer 1
BBB	Blood- brain- barrier
BCL-2	B- cell CLL/lymphoma- 2
BDNF	Brain- derived neurotrophic factor
BH	BCL- 2 homology domain
BID	BCL- 2- interacting domain death agonist
BIM	BCL- 2- interacting mediator of cell death
BSA	Bovine serum albumin
CDK5	Cyclin- dependent kinase 5
ChEI	Cholinesterase inhibitors
CNS	Central nervous system
CT	Computed tomography
DIV	Days in vitro
DLB	Dementia with Lewy's bodies
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	Deoxyribonucleic acid
ECE	Endothelin converting enzyme
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
ERK2	Extracellular signal- regulated kinase 2
E15	15^{th} day of pregnancy
FDA	Food and Drug Administration

FOXO	Forkhead box transcription factor
GAP	GTPase- activating protein
GSK3β	Glycogen synthase kinase- 3β
GTP	Guanosine triphosphate
HRP	Horseradish peroxidase
HT	Hormone therapy
IDE	Insulin- degrading enzyme
KRB	Krebs Ringer Buffer
LRP	Lipoprotein receptor- related protein
MAP	Microtubule- associated protein
MCL-1	Myeloid cell leukemia 1
mLST8	Mammalian lethal with SEC13 protein 8
MMP9	Matrix metalloproteinase 9
MMSE	Mini- Mental State Examination
MRI	Magnetic resonance imaging
MT	Mutant
mTOR	Mammalian target of rapamycin
mTORC2	Mammalian target of rapamycin complex 2
NEP	Neprilysin
NFT	Neurofibrillary tangles
NMDA	N- methyl D- aspartate
NP	Neuritic plaques
NSAID	Non- steroidal anti- inflammatory drug
PDK1	Phosphoinositide- dependent protein kinase- 1
PI	Phosphoinositide
PIP2	see PtdIns(4,5)P2
PIP3	see PtdIns(3,4,5)P3
PI3K	Phosphoinositide- 3- kinase
PKA	AMP(adenosine monophosphate)- dependent kinase
PKB	Protein kinase B, also known as AKT
PKC	Ca(Calcium)$^{2+}$- activated protein kinase
PKG	cGMP (cyclic guanosine monophosphate)- dependent kinase
PRAS40	Proline- rich AKT substrate 40kDa protein
PROTOR	Protein observed with RICTOR
PS1	Presenilin 1
PS2	Presenilin 2

PtdIns(3,4,5)P3	Phosphatidylinositol (3,4,5)- trisphosphate
PtdIns(4,5)P2	Phosphatidylinositol (4,5)- bisphosphate
RAGE	Receptor for advanced glycation end products
Rheb	Ras homology enriched in brain
RICTOR	Rapamycin- insensitive companion of mTOR
RSK	Ribosomal S6 kinase
SARM	Selective androgen receptor modulator
SDS	Sodium dodycyl sulfate
SDS- PAGE	SDS- polyacrylamide- gel electrophoresis
Ser	Serine
SERM	Selective estrogen receptor modulator
SIN1	SAPK (Stress-activated map kinase)-interacting protein 1
TSC1	Tuberous sclerosis 1 protein/ hamartin
TSC2	Tuberous sclerosis 2 protein/ tuberin
Thr	Threonine
TNFα	Tumor necrosis factor α
WHIMS	Women’s Health Iniative Memory Study
WT	Wild type

Contents

I LITERATURE REVIEW: Amyloid cascade hypothesis - many faces of Alzheimer's disease....10

1. Introduction.... 11
2. Etiology.... 15
3. Pathophysiology.... 16
   - 3.1 Formation of neuritic plaques (NP).... 17
   - 3.2 Formation of neurofibrillary tangles (NFT).... 20
   - 3.3 Synergism of Aβ and tau.... 20
   - 3.4 Toxic effects of microglia.... 21
4. Current therapies.... 23
   - 4.5 Cholinesterase inhibitors (ChEIs).... 23
   - 4.6 N-methyl-D-aspartate (NMDA) antagonist.... 25
5. Future therapies.... 26
   - 5.1 Targeting Aβ.... 26
     - 5.1.1 Formation.... 26
     - 5.1.2 Clearance and degradation.... 27
   - 5.2 Sex hormones.... 27

II EXPERIMENTAL PART: Effect of impaired AKT function on phosphorylation of pro- apoptotic BAD....31

6. Introduction.... 32
   - 6.1. AKT.... 32
   - 6.2. Activation of AKT.... 32
   - 6.3. AKT substrates.... 34
     - 6.3.1. TSC2.... 34
     - 6.3.2. PRAS40.... 36
     - 6.3.3. GSK3.... 36
     - 6.3.4. FOXO.... 36
     - 6.3.5. BAD.... 37
   - 6.4. PDK1 K465E- mutant mice.... 40
   - 6.5. Aims of the study.... 41

7. Materials and methods 42
7.1. Cell culture and stimulation 42
7.2. The Bradford assay 45
7.3. Immunoprecipitation 45
7.4. Preparing the samples for Western Blot 46
7.5. Western blot 47
7.5.1. SDS- PAGE 47
7.5.2. Electroblotting 49
7.5.3. Antibody-antigen based detection 50
8. Results and discussion 52
8.1. Effect of immunoprecipitation 52
8.2. Antibodies from different suppliers 55
8.3. PDK1 K465E neurons 56
9. Conclusions 59
10. References 61

I Literature review:

Amyloid cascade hypothesis– many faces of Alzheimer's disease

1. Introduction

The most common cause to dementia on elderly is Alzheimer's disease (AD) (Knopman 2006, Rinne and Koulu 2007, Färkkilä and Pirttilä 2011, Hong-Qi et al. 2012). It covers over 60% of all dementias when the percentage of others, for example vascular dementias or Dementia with Lewy bodies (DLB) is significantly lower. Although AD is sometimes used incorrectly to describe all the dementias in the elderly, the disease has its own diagnostic criteria that distinguish it from other forms.

Clinical diagnosis requires over six months of memory decline accompanied by problems with self-care and social or occupational functions (Chu 2012). In most cases, family members observe these changes first and seek medical care for patient. Accurate diagnosis requires several aspects, including information of family history, age, several cognitive tests that measure memory, language skills and person's abilities to function in everyday life. Memory is usually tested with MMSE (Mini-Mental State Examination) –test (figure 1) (Yeoman et al. 2006, Chu 2012). MMSE scores between 24 and 10 indicate mild-to-moderate AD, respectively and scores lower than 10 indicate severe AD.

It is also important to exclude other brain diseases if they are suspected, by using either computed tomography (CT) or magnetic resonance imaging (MRI). There are also several other conditions that can cause dementia and must first be ruled out, for example hypothyroidism, neurosyphilis, sedation from drugs, chronic heavy metal intoxication, human immunodeficiency virus (HIV) infection and Creutzfeldt- Jakob disease.

The mini mental state examination

Orientation

Year, month, day, date. season	_____/5
Country, county, town, hospital, ward (clinic)	_____/5

Registration

Examiner names three objects (for example, apple, pen, and table)	
Patient asked to repeat objects, one point for each.	_____/3

Attention

Subtract 7 from 100 then repeat from result, stop after five subtractions. (Answers: 93, 86, 79, 72, 65)	
Alternatively if patient errs on subtraction get them to spell world backwards: D L R O W	
Score best performance on either task.	_____/5

Recall

Ask for the names of the objects learned earlier.	_____/3

Language

Name a pencil and a watch.	_____/2
Repeat: 'No ifs, and or buts.'	_____/1
Give a three stage command. Score one for each stage (for example, 'Take this piece of paper in your right hand, fold it in half and place it on the table.'	_____/3
Ask patient to read and obey a written command on a piece of paper stating: 'Close your eyes.'	_____/1
Ask patient to write a sentence. Score correct if it has a subject and a verb.	_____/1

Copying

Ask patient to copy intersecting pentagons.	
Score as correct if they overlap and each has five sides.	_____/1

Total score: _____/30

Figure 1. MMSE-test is basic tool used in clinical diagnosis of AD to measure memory impairment (Yeoman et al. 2006).

Clinical characteristics of the disease are progressive disturbances in memory and especially in recent memory and in new learning (Knopman 2006, Yiannopoulou and Papageorgiou 2013). Deficit in cognition leads to other symptoms including disorientation of time and place, impaired judgment and problem- solving and disturbances of language skills. These key manifestations of AD are typically followed by varying neuropsychiatric symptoms such as depression, agitation and sleep disturbances. Progression of the disease lead eventually to death, depending of the individual in nine to twelve years from onset of symptoms (Knopman 2006). Some approximate milestones of AD are shown in the figure 2.

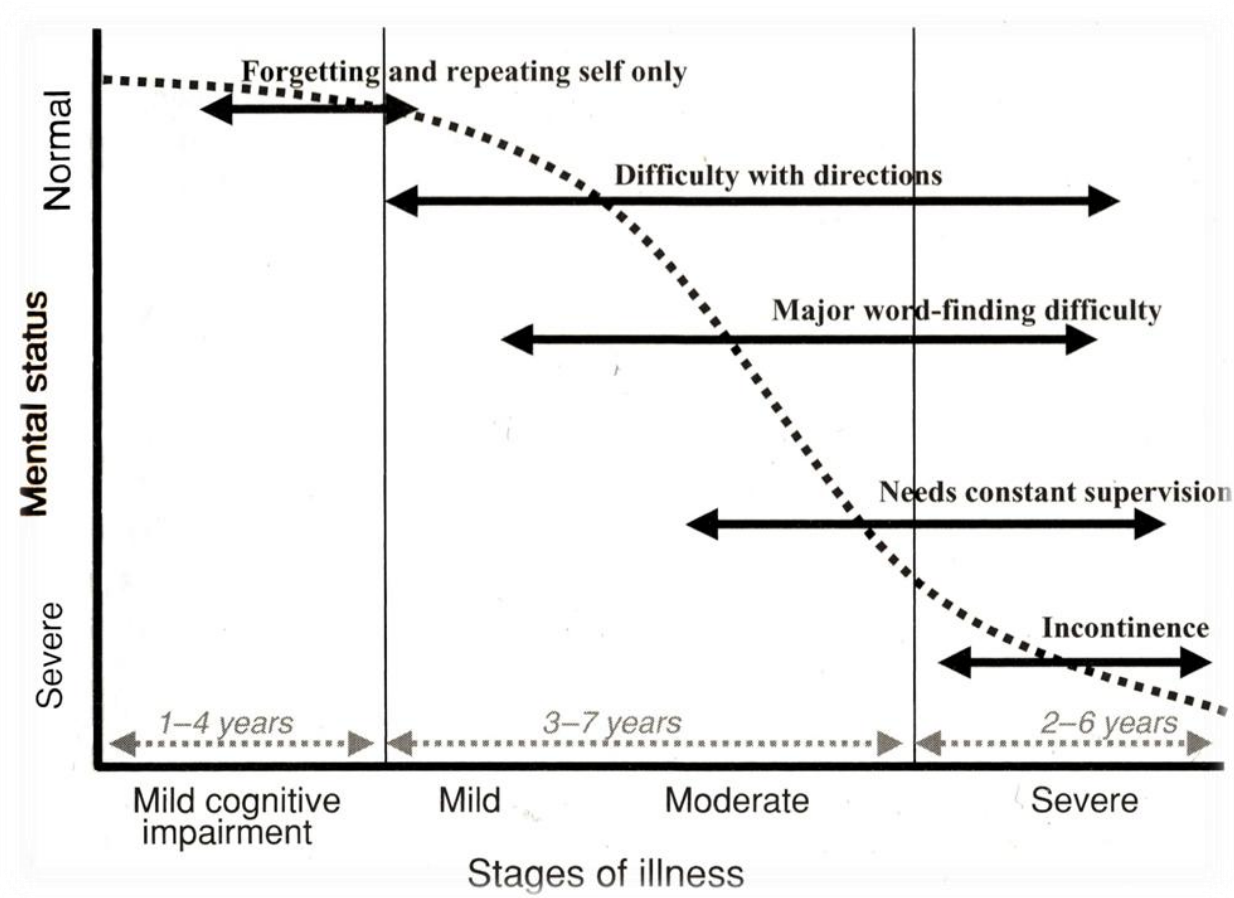


Figure 2. Progression of Alzheimer's disease (Knopman 2006).

It was estimated that in 2007 in Finland 100 000 people had moderate or severe AD (Färkkilä and Pirttilä 2011). The prevalence of AD correlates with advancing age (Knopman 2006, Rinne and Koulu 2007, Färkkilä and Pirttilä 2011, Gauthier et al. 2012, Hong-Qi et al. 2012, Povova et al. 2012). AD is rare under the age of 60 whereas it is estimated that 20- 50% of over the age of 85 has it. As the population ages, the prevalence of AD grows. It is believed that the prevalence doubles every 20 years. Everyone has the major risk factor: age, if living long enough.

2. Etiology

Two forms of the disease have been recognized, familial and sporadic Alzheimer's disease (Lippa et al. 1996, Knopman 2006). There are three genes that have been implicated in familial forms of AD: amyloid precursor protein (APP), presenilin- 1 (PS1) and presenilin- 2 (PS2) and they all are autosomal dominant genes (Selkoe 1999, Knopman 2006, Chu 2012). Mutations in APP, PS1 or PS2 gene favors formation of amyloidogenic Aβ- peptides by β- and γ- secretases (see chapter 3.1.) (Kimberly et al. 2000, Hardy and Selkoe 2002). Especially mutation in PS1 gene, which is the catalytic core of γ- secretase, increases $A\beta_{42}$: $A\beta_{40}$ ratio (Placanica et al. 2009).

Inheritance of one or two alleles of susceptibility gene apolipoprotein E (ApoE), also increases the risk for developing AD in both forms of the disease.

Familial and sporadic forms of the disease lead to same end point and share same pathologic features, although in familial form onset of symptoms happen on earlier age and histological changes are more severe (Lippa et al.1996). However, familial AD is rare (Chu 2012). Approximately 15% of AD patients have family history of the disease.

3. Pathophysiology

The key event in pathogenesis of AD is altered processing and deposition of two proteins: β- amyloid (Aβ) and tau (Knopman 2006, Querfurth and LaFerla 2010, Apostolova et al. 2012). Accumulation of these proteins to the brain leads to dysfunction and eventually to death of neurons. Other pathological features are amyloid angiopathy, oxidative and inflammatory damage and loss of white matter due to the loss of axons.

Typical areas in the brain where atrophy can be detected in AD patients are cortex and hippocampus, accompanied with enlargement of cerebral ventricles (Apostolova et al. 2012). Some of these features are associated with normal aging, but with far slower progression rate. Atrophy has an impact especially on cholinergic system (figure 3), starting from basal forebrain and leading to cortex and limbic areas of the brain (Farlow 2002). This leads to progressive loss of acetylcholine neurons and impaired production and degradation of acetylcholine (Chu 2012, Yiannipoulou and Papageorgiou 2013). Loss of cholinergic activity correlates with cognitive dysfunction, and it declines as the disease progress (Farlow 2002).

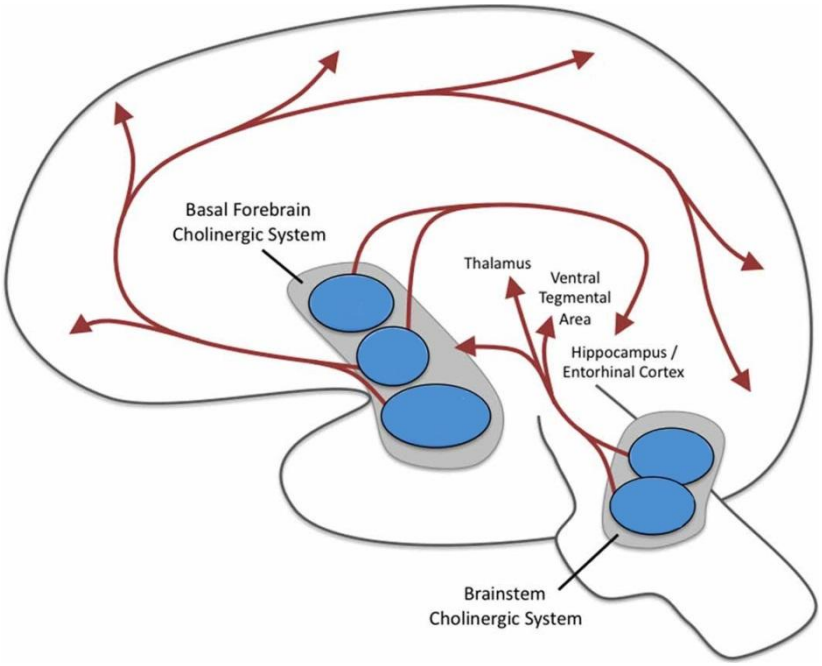


Figure 3. Cholinergic system in the brain (Revised from Newman et al. 2012).

Progressive atrophy involves also other neurotransmitter systems, for example it causes imbalance in monoamine and excitatory amino acid systems (Farlow 2002). One important neurotransmitter that is affected is glutamate. Glutamate is excitatory neurotransmitter that mediates learning and memory in pyramidal cells in the hippocampus and cortex via N-methyl D-aspartate (NMDA) receptors (Chu 2012). Although an excess amount of glutamate is detrimental and it is associated with neurotoxicity.

3.1 Formation of neuritic plaques (NP)

APP is cleaved by three secretases, α, β and γ (Kimberly et al. 2000, Knopman 2006, Querfurth and LaFerla 2010, Hong-Qi et al. 2012, Yiannopoulou and Papageorgiou 2013). Aβ- peptides are natural metabolic end products deprived by β- secretase, also named β-site APP- cleaving enzyme 1 (BACE1), and γ-secretase. β- and γ-secretase are responsible for formation of amyloidogenic peptides and α- secretase proteolysis leads to formation of nonamyloidogenic end products (figure 4). Localization of amyloid precursor protein (APP) to chromosome 21 was supported by the earlier findings that Down's syndrome (trisomy 21) has link with AD (Hardy and Selkoe 2002, Knopman 2006). It was found that possessing extra chromosome 21 with the APP gene accelerated the pathology of AD (even 30 years) in Down's syndrome patients.

Aβ- peptides consist of 36 to 43 amino acids and corresponding to the major cleaving sites of γ- secretase (Kimberly et al. 2000, Knopman 2006, Querfurth and LaFerla 2010, Hong-Qi et al. 2012, Yiannopoulou and Papageorgiou 2013). $A\beta_{40}$ and $A\beta_{42}$ are the most common forms. $A\beta_{42}$ species are found to be the most harmfull due to its tendency to aggregate into fibrils and thus trigger the formation of neuritic plaques (Tanzi and Bertram 2005). As the figure 5 shows, first phases of the amyloid cascade hypothesis comprise of overproduction and accumulation of Aβ and formation of plaques, leading progressively to dementia (Hardy and Selkoe 2002).

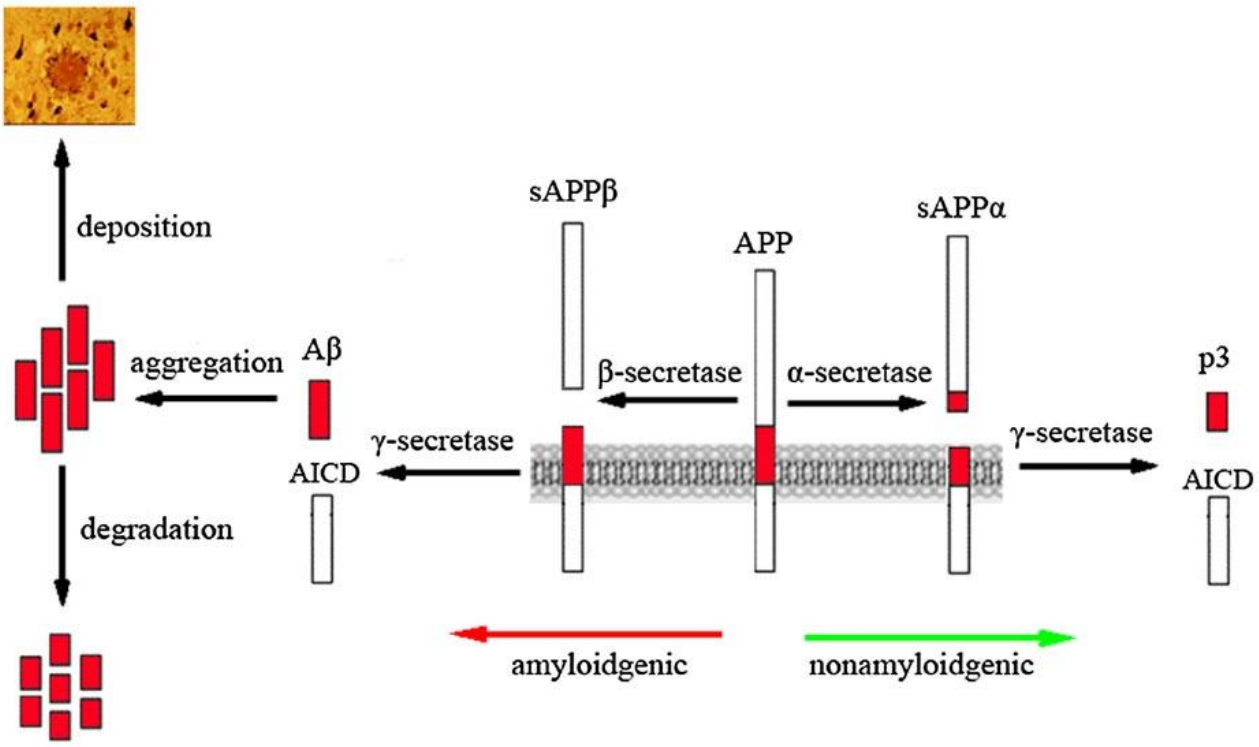


Figure 4: Processing of Amyloid Precursor Protein (Revised from Hong-Qi et al. 2012). AICD = amyloid intracellular domain

Several researches reveal that the total amount of amyloid plaques found post-mortem from the AD patients' brain does not always correlate to the severity of the disease (Querfurth and LaFerla 2010, Selkoe 2011). Still it is belived that among individuals who possess mutations in APP or presenilin genes, the causative reason for AD is accumulation and aggregation of $A\beta_{42}$ (Selkoe 2011).

Rising of the Aβ monomer levels caused by several reasons, slowly leads to formation of dimers and then larger oligomers (Selkoe 2011). With time, these molecules start to form progressively growing fibrils with a tendency to bind cell membranes. This event temporarily decreases the amount of free particles in the brain and thus can be seen as protective phase. As the disease progress, fibrils develop to plaques eventually reaching maximal size and density, allowing increasing amount of oligomers to remain free to cause synaptic/ neuritic injury to surrounding cells. So the combined effect of neuritic plaques and free diffusible oligomers are the cause of Aβ induced neuronal death.

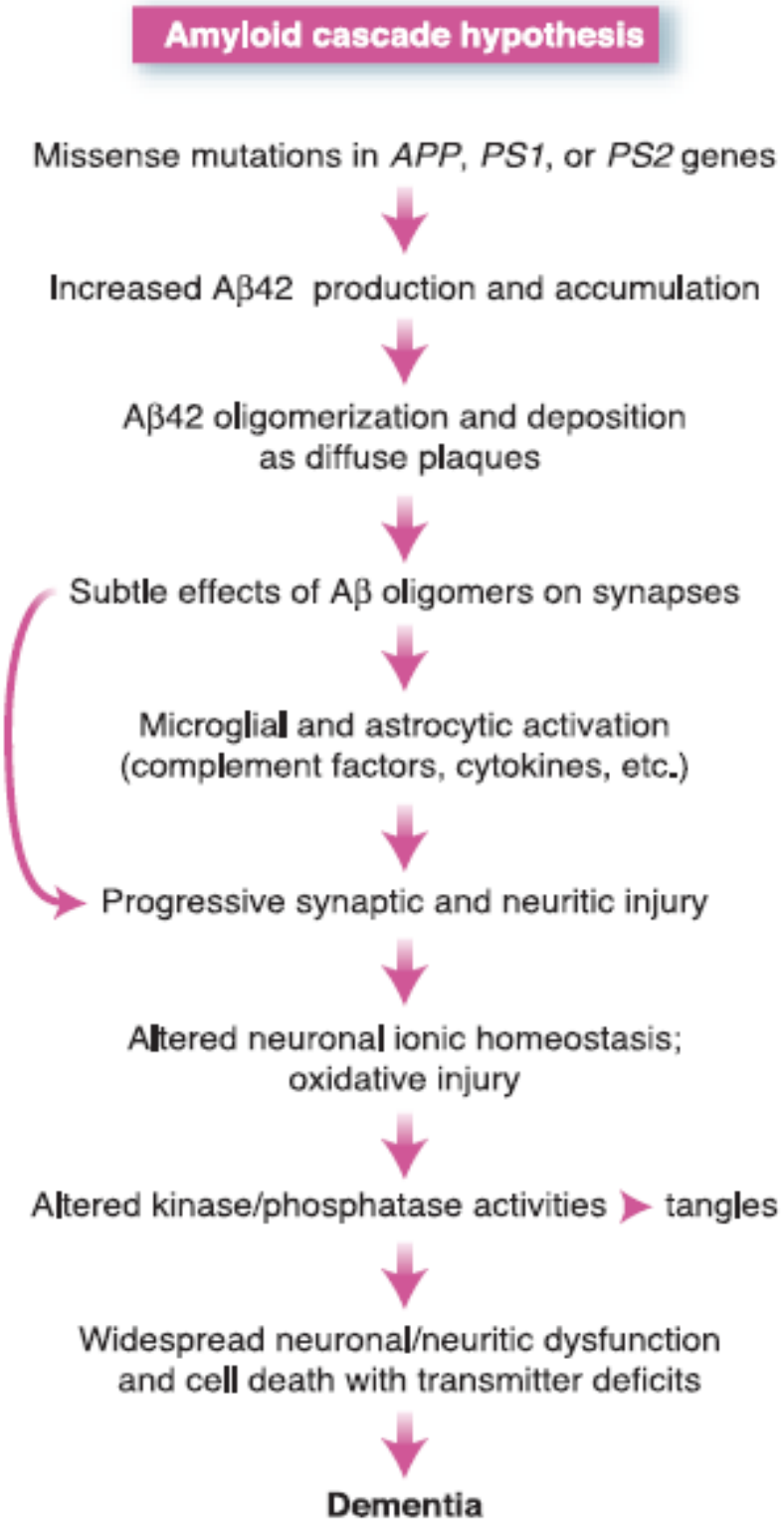


Figure 5. Amyloid cascade hypothesis (Hardy and Selkoe 2002).

3.2 Formation of neurofibrillary tangles (NFT)

Tau is a microtubule- associated protein (MAP) that resides mainly in the axons (Querfurth and LaFerla 2010, Duan et al. 2012). In normal conditions it is a soluble protein which is controlled by balance between phosphorylation and dephosphorylation. The primary function of tau is to maintain stability of microtubules and it is also responsible for the transport of signalling molecules, trophic factors and other essential cellular constituents (for example mitochondrias and vesicles) along the axons.

When dephosphorylation is impaired, tau becomes hyperphosphorylated by several serine/ threonine kinases and amount of free tau fraction is elevated (Duan et al. 2012). When tau is hyperphosphorylated it becomes insoluble and it detaches from microtubules. Hyperfosforylated tau form pretangles (nonfibrillary tau deposits), paired helical filaments and finally combination of these leads to formation of neurofibrillary tangles (NFTs).

Several kinases responsible for tau phosphorylation have been suggested, for example insulin- mediated AKT substrate glycogen synthase kinase 3β (GSK3β) (see II EXPERIMENTAL PART chapter 6.3.3.), cyclin- dependent kinase 5, (CDK5) and extracellular signal- regulated kinase 2 (ERK2) (Zhao and Townsend 2009, Duan et al. 2012, Hong-Qi et al. 2012, Tokutake et al. 2012, Yiannopoulou and Papageorgiou 2013).). If AKT signaling is impaired, it is reflected by weakened inhibition of GSK3β. This leads to overactivation of GSK3β and thus to hyperphosphorylation of tau (Zhao and Townsend 2009). Controlling the overactivity of these kinases may show potential in disease-modifying therapies in the future.

3.3 Synergism of Aβ and tau

Connection between Aβ and tau is not yet fully elucidated, but it is believed that accumulation of Aβ initiates tau hyperphosphorylation and vice versa, tau can mediate Aβ toxicity (Querfurth and LaFerla 2010, Duan et al. 2012, Parthsarathy et al. 2013). According to the research made by Götz et al. (2001), $A\beta_{42}$ fibrils enhance NFT formation in vivo supporting the hypothesis that causative factor to pathogenesis

is Aβ. Furthermore in the research of Tokutake and coworkers (2012) was suggested that elevated Aβ- levels in the AD brain disrupt the insulin- PI3- AKT signaling (see chapter 6.2.) which leads to tau hyperfosforylation by GSK3β. On the other hand, the research of Rapoport et al. (2002) provides evidence that tau has a key role in the mechanism that leads to fibrillar Aβ- deposits.

3.4 Toxic effects of microglia

Microglias are considered as brain's macrophages (Venneti et al. 2009, Rosenberg 2005). They are important immune cells capable of phagocytosis and antigen presentation. They become activated by several factors that pose a potential threat to homeostasis of the brain. One of those factors is Aβ (Rogers et al. 2002, Rosenberg 2005). Accumulation of Aβ initiates inflammation and neurotoxic process (Selkoe 1999).

Aβ-plaques attract microglia to their vicinity where they become activated. Activated microglia has ability to phagocytose Aβ-plaques, and interestingly estrogen is found to increase this ability (Rogers et al. 2002, Rosenberg 2005). Microglia also produce cytokines, neurotoxins and some proteolytic enzymes that degrade Aβ, such as IDE (insulin-degrading enzyme), NEP (neprilysin), MMP9 (matrix metalloproteinase 9) and plasminogen.

The role of microglia in AD pathogenesis is controversial (Venneti et al. 2009). It is known that microglia are capable to phagocytose amyloid plaques and soluble Aβ and therefore enhance amyloid clearance (Rogers et al. 2002, Venneti et al. 2009). It has also been demonstrated that microglia have important role of promoting adult neurogenesis. It is normal that neurogenesis declines with age, but it is especially disrupted with AD patients.

Taken these protective mechanisms together a question arises: what causes microglia to change from anti-inflammatory/ neuroprotective to pro-inflammatory/ neurotoxic mediators?

The research made by Hickman et al. (2008) indicates that microglia become dysfunctional as the disease progress and the Aβ load increases. Due to the

reduction of Aβ- binding receptors microglia lose the ability to phagocytose Aβ and also production of Aβ- degrading enzymes reduces significantly. However, the ability to produce pro- inflammatory cytokines maintains. Interestingly, cytokines have been found to be responsible of reduction of Aβ- binding receptors and reduced expression of proteolytic enzymes. It has also been found that the main cytokines produced by microglia, TNFα (tumor necrosis factor α) and several others, stimulate β- secretase (BACE1) and γ- mediated cleavage of APP therefore increasing the Aβ load even further.

4. Current therapies

Despite of decades of active research, there is still no curative treatment for AD (Hong-Qi et al. 2012, Huang and Mucke 2012, Yiannopoulou and Papageorgiou 2012). According to the figure 5 and generally accepted theory, the accumulation of Aβ is the key event in AD pathogenesis. Thus a lot of time and effort have been spent to develop therapeutics that reduce Aβ load in the brain (Ghosh et al. 2012).

Overall, there are several potential steps in the pathogenesis of AD that could be targeted with therapies (Hardy and Selkoe 2002, Huang and Mucke 2012, Yiannopoulou and Papageorgiou 2013). In addition to Aβ accumulation and NFTs, also inflammation, oxidative damage, deregulation of metals, mitochondrial impairments and cholesterol metabolism are responsible of development of clinical symptoms.

All current treatments to Alzheimer's disease are symptomatic neutransmitter enhacement therapies (Chu 2012, Yiannopoulou and Papageorgiou 2013). They do not modify or stop the course of the disease but they alleviate symptoms and perhaps postpone institutionalization of an individual.

4.5 Cholinesterase inhibitors (ChEIs)

Cholinesterase inhibitors prolong availability and activity of acetylcholine in the synaptic cleft by reducing its hydrolysis by acetylcholinesterase (and additionally butyrylcholinesterase by rivastigmine) (Farlow 2002, Chu 2012). First ChEI approved by U.S. Food and Drug Administration (FDA) for AD was tacrine in 1993 but it is not used anymore due to hepatotoxicity (Farlow 2002, Chu 2012, Hong- Qi et al.2012, Yiannopoulou and Papageorgiou 2013). Currently there are three cholinesterase inhibitors (ChEIs) approved for clinical use of mild to moderate AD (MMSE between 24-10; figure 1): donepezil, rivastigmine and galantamine (approved by FDA in 1996, 2000 and 2001, respectively). Donepezil has later approved also for the treatment of severe AD (MMSE <10; figure 1) in the USA.

ChEIs are standard first-line drugs used to treat AD (table 1). Several reviews and meta-analyses indicate improvement in cognitive functions and MMSE scores over the first three months with ChEI use compared to placebo. Also long-term treatments (up to five years) have showed delayed decline in cognitive functions and activity of daily living.

Table 1. Current therapies used in AD. (Revised from Chu 2012, Pharmaca Fennica 2013).

Drug	Class	Dosage form	Dose (mg/day)	Frequency (times/day)	Metabolism
Donepezil	ChEI	Oral	5-10	1	CYP2D6 CYP3A4
Galantamine	ChEI	Oral	8-24	1*	CYP2D6 CYP3A4
Rivastigmine	ChEI	Oral, transdermal patch	3-12	2**	Non-hepatic
Memantine	NMDA-receptor antagonist	Oral	5-20	2	Non-hepatic

* When used prolonged-release formulation
** With transdermal patch change once a day

Generally ChEIs are well tolerated (Chu 2012). Typical adverse events of ChEIs are gastro-intestinal, such as nausea, vomiting, diarrhea and abdominal cramp (Chu 2012, Yiannopoulou and Papageorgiou 2013). Systemic reviews suggests that the incidence is lower with donepezil that with rivastigmine and galantamine. Adverse events are more common with higher therapeutic doses and during dose escalation. Therefore slow titration upwards is in key role when starting ChEI treatment. Table 1 shows starting- maximum doses of each ChEI. As table 1 indicates, all ChEIs are available in oral forms, but only rivastigmine has transdermal patch formulation. Use of transdermal rivastigmine minimizes gastro-intestinal adverse events, so it is good option for oral formulations especially if patient suffers from intense side effects.

Comparison of efficacy between ChEIs is poorly documented and limited evidence exists (Chu 2012). If a patient does not respond to one ChEI, there is an option of switching to an alternate ChEI with a different pharmacologic profile (Farlow 2002). Especially if patient is treated unsuccessfully with selective acetylcholinesterase inhibitor (donepezil or galantamine) may benefit of switching to rivastigmine which is dual inhibitor of acetyl- and butyrylcholinesterase.

4.6 N-methyl-D-aspartate (NMDA) antagonist

Moderate- affinity non- competitive, NMDA- type receptor antagonist memantine was approved for treatment of moderate to severe AD by FDA in 2003 (Chu 2012, Hong-Qi et al. 2012, Yiannopoulou and Papageorgiou 2013). As the disease progress, other neurotransmitter systems are also affected (see chapter 3.) and excess amount of glutamate is present. Memantine is believed to protect neurons from glutamate induced excitotoxicity while maintaining normal concentration of glutamate needed on learning and memory functions.

Clinical trials have shown beneficial effects of memantine compared to placebo on cognition, behavior and activity of daily living (Chu 2012, Hong-Qi etc. 2012, Yiannopoulou and Papageorgiou 2013). Use of memantine indeed alleviates deterioration of mood, agitation, irritability or delusions.

Memantine treatment is recommended to start with the dose 5 mg once a day (Chu 2012). After this it is increased weekly by 5 mg up to maximum daily dosage of 20 mg. Overall memantine is well tolerated. Putative adverse events are hardly more common compared to placebo-treated patients. Dizziness, confusion, hallucinations, head ache, somnolence and nausea are the most common adverse events reported (Chu 2012, Yiannopoulou and Papageorgiou 2013).

Dual therapy with memantine and donepezil with moderate or severe AD patients have shown considerable improvement in cognitive and functional outcomes, activity of daily living and behavior compared with placebo group (memantine and placebo) (Hong-Qi et al.2012, Yiannopoulou and Papageorgiou 2013).

5. Future therapies

5.1 Targeting Aβ

As it is known, Aβ is a neurotoxin and its accumulation to the brain leads to oxidative stress and neuronal dysfunction and destruction (Hong-Qi et al. 2012). Preventing formation and accumulation of Aβ in the brain is in the key role on the road to developing therapeutics.

5.1.1 Formation

β- and γ- secretases, which are responsible of Aβ- formation, are interesting targets of drug design (Hardy and Selkoe 2002, Tanzi and Bertram 2005, Hong- Qi et al. 2012, Huang and Mucke 2012, Yiannopoulou and Papageorgiou 2013). One therapeutic approach could be inhibiting these secretases and thus prevent excessive Aβ- formation. However, development of β- and γ- secretase inhibitors has faced many problems including a weak bioavailability, a poor blood- brain- barrier (BBB)- penetration and difficult side effects.

Typical problems with β- and γ- secretase inhibitors are detrimental side effects, because their diverse regulation of other substrates (Hardy and Selkoe 2002, Tanzi and Bertram 2005, Hong- Qi et al. 2012, Huang and Mucke 2012, Yiannopoulou and Papageorgiou 2013). One γ- secretase substrate is Notch receptor 1, an important protein involved in growth and development. Inhibition of Notch leads to severe gastrointestinal and hemopoetic side effects and this has been an obstacle hampering the development of clinically potent γ- secretase inhibitor.

Complications encountered with BACE1 inhibitors mostly result from inadequate BBB- penetration (Hardy and Selkoe 2002, Tanzi and Bertram 2005, Hong- Qi et al. 2012, Huang and Mucke 2012, Yiannopoulou and Papageorgiou 2013). The drugs developed have been mostly small peptides that have to be administered directly to the brain to achieve effects and therefore their clinical use seems unpromising.

Two γ- secretase inhibitors have made to the phase III trials but failed: a putative γ-secretase modulator R- flurbiprofen/ tarenflurbil derived from a NSAID (non- steroidal anti- inflammatory drug) and γ- secretase inhibitor semagestat (LY- 450139) (Selkoe 2011, Hong-Qi et al. 2012, Yiannopoulou and Papageorgiou 2013). Setbacks were due to a low efficacy, a poor brain penetration and severe side effects, respectively.

5.1.2 Clearance and degradation

One reason for elevated Aβ levels in the brain is impaired clearance and degradation of Aβ (Tanzi and Bertram 2005, Hong-Qi et al. 2012). Low- density lipoprotein receptor- related protein (LRP) plays a key role in exporting Aβ from the brain. If the function of LRP is impaired it reduces Aβ clearance substantially. It has been found that in AD patients LRP activity is lowered (Tanzi and Bertram 2005). In contrast a protein responsible for transporting Aβ in to the brain (the receptor for advanced glycation end products/ RAGE) has become more active in AD patients. Affecting to the balance between these transporters may show therapeutic potential, but no clinical data is yet available.

Major degrading enzymes that participate in Aβ clearance are neprilysin (NEP) and insulin degrading enzyme (IDE) (Tanzi and Bertram 2005, Querfurth and LaFerla 2010, Hong-Qi et al. 2012). Animal models have shown that inhibition of these enzymes decrease Aβ degradation and decline cognitive activity (Hong-Qi et al. 2012). Also endothelin converting enzyme (ECE), 1 and 2 angiotensin- converting enzyme (ACE) and plasminogen system have the ability to degrade the Aβ- peptide at different amino acid residues within the Aβ sequence.

5.2 Sex hormones

Female sex steroid hormones are mainly produced in the ovary but it has been demonstrated that also glial cells and neurons are capable of producing them in several regions in the central nervous system (CNS) and especially in the hippocampus (Carroll and Rosario 2012).

Estrogens and androgens (male sex steroid hormones) are found to be neuroprotective agents and they promote neural viability and facilitate synaptic plasticity in the brain (Barron and Pike 2012, Carroll and Rosario, Hong-Qi et al. 2012). It has been demonstrated that estrogens and androgens regulate production and clearance of Aβ, and they also reduce Aβ levels in the brain (Barron and Pike 2012). Therefore it has been hypothesized that age- related loss of these hormones, especially with post- menopausal women, represents an important risk-factor to AD.

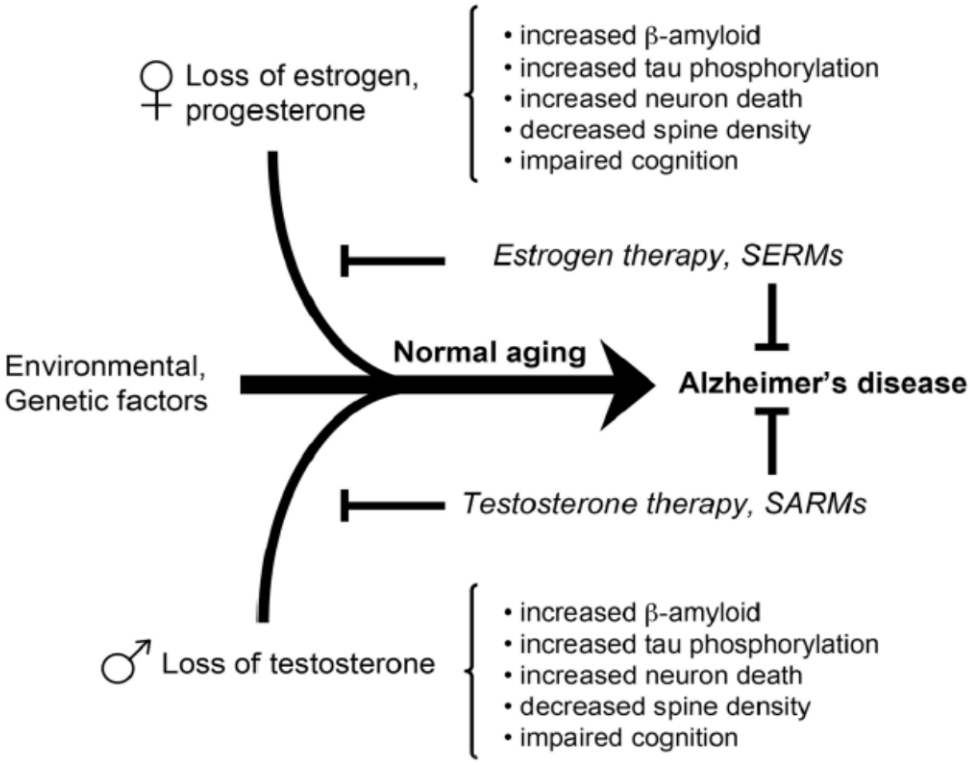


Figure 6. A schematic view of different risk factors affecting AD, and possible sex hormone- based therapies (Barron and Pike 2012).
SARM/ SERM = Selective Androgen/ Estrogen Receptor Modulator

Similar to the estrogen, age- related testosterone loss in men is also connected with the increased risk of developing AD (Carroll and Rosario 2012). Although testosterone levels decrease along with age, there is a deal of variability among individuals. Androgens have several beneficial actions in the brain, which is a highly androgen- responsive tissue. Older men with high levels of free circulating

testosterone have shown to exhibit better long- term memory (Carroll and Rosario 2012).

Results of hormone replacement therapies in the treatment of AD are controversial. Some researches indicate that hormone therapies have therapeutic efficacy by improving memory and cognition in older postmenopausal women with AD (Wharton et al. 2011). However, Women's Health Initiative Memory Study (WHIMS) done by Shumaker et al. (2003, 2004) indicates that hormone therapy by using estrogen plus progestin or estrogen alone increases the risk of dementia and impairment of cognition. Hormone replacement therapy use is also associated with adverse events, such as promoting cancers in reproductive tissues or in breasts (Shumaker et al. 2003, Barron and Pike 2012, Carroll and Rosario 2012).

Hormone replacement therapy is rare with men, although it is estimated that 30%-70% of men over 70 years in the U.S. suffers from androgen deficiency (Carroll and Rosario 2012). Hormone replacement therapy in preventing cognitive decline in aging men is far less studied compared to post- menopausal women. Few results of efficacy of hormone replacement therapy use in treating and or preventing AD in aged men provide mixed results. As with estrogen- based therapy of aging women, risks linked with testosterone therapy include different cancers, atherosclerosis and hypertension.

Now efforts has been targeted to develop selective estrogen and androgen receptor modulators (SERM and SARM) that are able to penetrate BBB, has neuroprotective effects and do not have receptor- dependent effects on other hormonal tissues (breasts, uterus, prostate) (Carroll and Rosario 2012).

As figure 7 shows, sex hormones and especially androgen has many beneficial effects related to AD pathogenesis. Targeting these mechanisms with proper SARMs may turn out as future therapy in reducing the risk of AD in aging men.

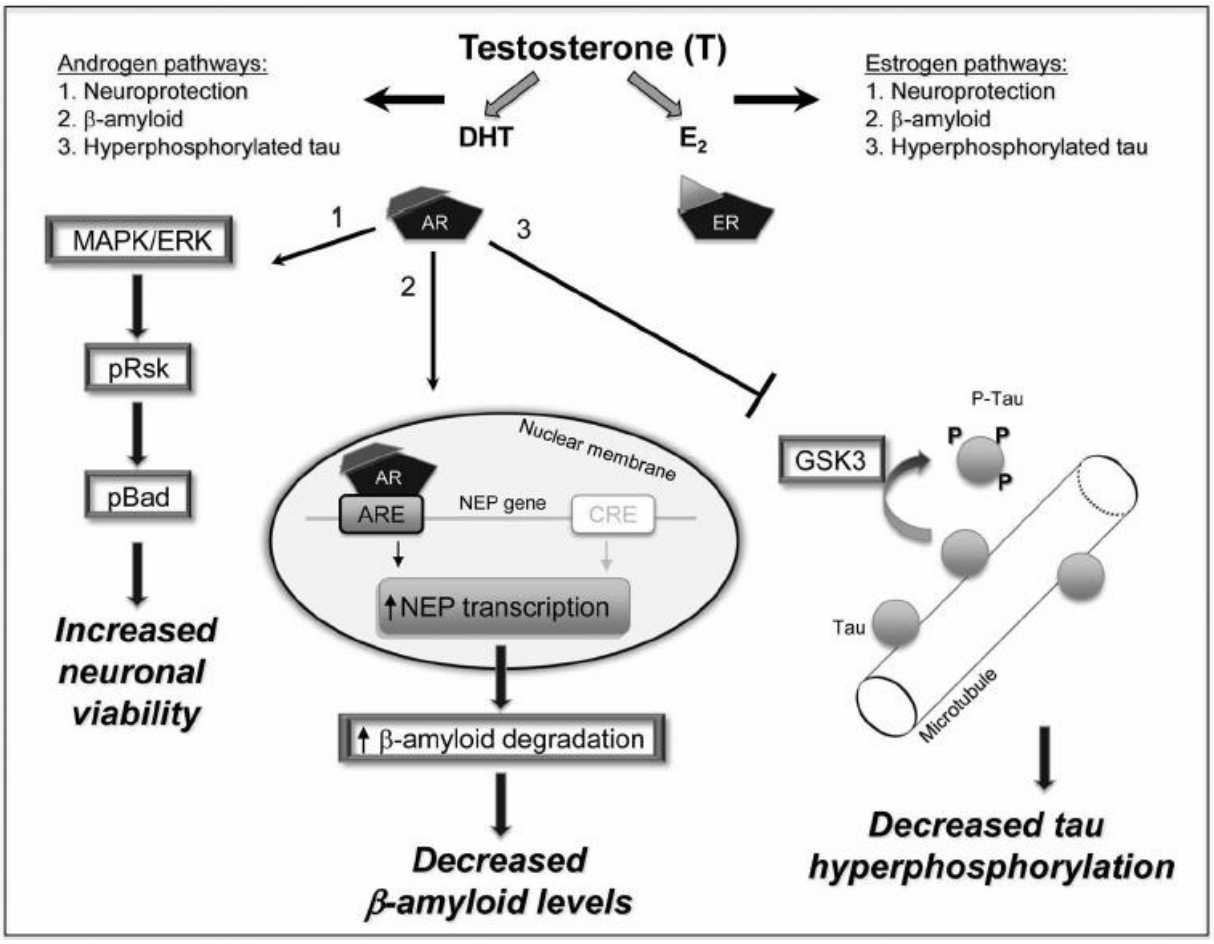


Figure 7. Many effects of sex hormones (Carroll and Rosario 2012). Androgen is known to mediate many pathways related to AD by: 1. increasing neuronal viability via promoting phosphorylation of pro- apoptotic kinase Bad (see II EXPERIMENTAL PART chapter 6.5.3.) 2. decreasing Aβ- levels by inducing degrading enzyme neprilysin (NEP) and 3. decreasing tau hyperphosphorylation by inhibiting GSK3β (see II EXPERIMENTAL PART chapter 6.3.3.).

II EXPERIMENTAL PART:

Effect of impaired AKT function on phosphorylation of pro- apoptotic BAD

6. Introduction

6.1. AKT

Protein kinase B (PKB) also known as AKT (figure 8), is a serine/threonine protein kinase that mediates many important downstream events regulated by phosphoinositide-3-kinase (PI3K) (Lawlor and Alessi 2001). Akt gene family has three members named as AKT1 (PKBα), AKT2 (PKBβ) and AKT3 (PKBγ) (Bellacosa et al. 2004). Tissue distribution of different members varies, where AKT1 and AKT2 are expressed ubiquitously while AKT3 is mainly found in neuronal tissues.

AKT family belongs to the class of AGC kinases. This name comes from the members of the family, namely AMP-dependent kinase (PKA), cGMP-dependent kinase (PKG) and Ca^{2+}-activated protein kinase (PKC) as well as phosphoinositide-dependent protein kinase-1 (PDK1) (Lawlor and Alessi 2001, Parker and Parkinson 2001, Hanada et al. 2004). All of these protein kinases are activated and regulated by small second messengers such as cyclic mononucleotides, Ca^{2+} or phosphoinositides.

All three AKT isoforms share a similar structure (Hanada et al. 2004, Vasudevan and Garraway 2010). They consist of different functional domains including an amino terminal pleckstrin homology (PH) domain, a central kinase domain and the hydrophobic motif, a carboxyl-terminal regulatory domain that is a representative of AGC kinase-family.

6.2. Activation of AKT

AKT is activated downstream by the phosphoinositide-3-kinase (PI3K), in response to growth factors, cytokines and hormones such as insulin (Figure 8) (Lawlor and Alessi 2001, Bellacosa et al. 2004, Pearce et al. 2010, Vasudevan and Garraway 2010). After PI3K is recruited to the plasma membrane and activated, it phosphorylates 3´-OH position in the inositol phospholipids to 3´-phosphoinositides

(PIs) (Vivanco and Sawyers 2002). These important second messengers generated by PI3K are called phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5) P2 or PIP2)) and phosphatidylinositol (3,4,5)- trisphosphate (PtdIns(3,4,5)P3 or PIP3).

PH is the domain in the N-terminal region of AKT that interacts with second messengers PIP2 and especially with PIP3 (Bellacosa et al. 2005, Hennessy et al. 2005). This event contributes to the recruitment of AKT to the plasma membrane and triggers a conformational change that is vital for phosphorylation at two main amino acid residues of AKT.

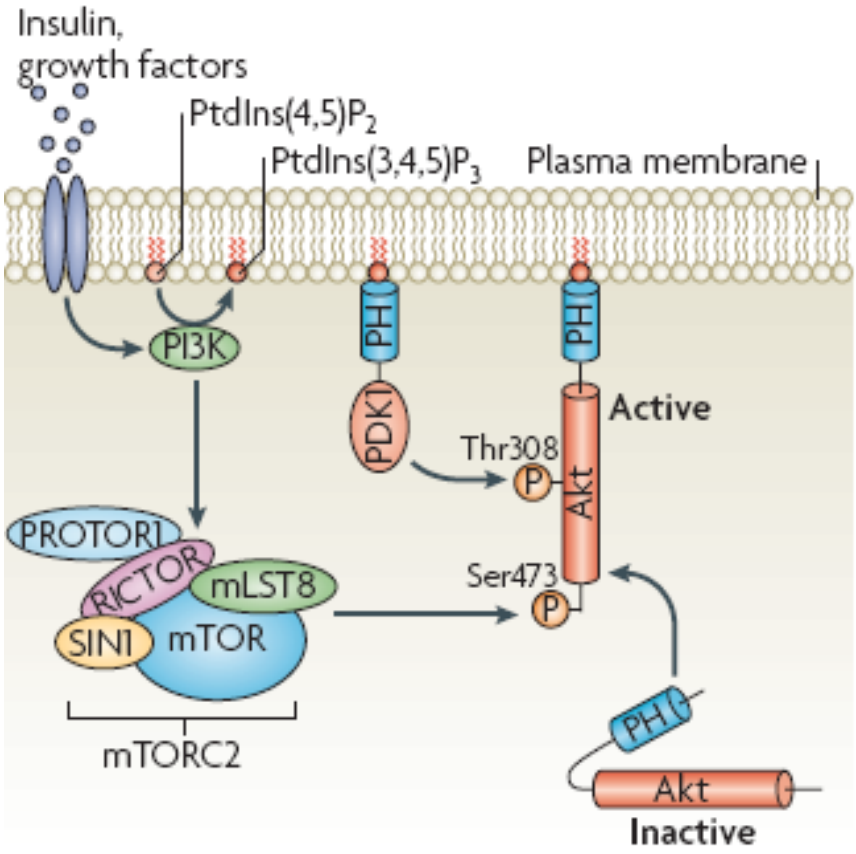


Figure 8. Phosphorylation of AKT (Pearce et al. 2010).

One activating residue is threonine- 308, and it resides in the central kinase domain (Sarbassov et al. 2005, Vasudevan and Garraway 2010, Hers et al. 2011). Thr- 308 phosphorylation occurs via phosphoinositide- dependent protein kinase- 1 (PDK1). PDK1 can phosphorylate itself at position Ser-241 leading to its activation

(Casamayor et al. 1999). As well as its substrate AKT, PDK1 contains PH domain which interaction with PIP3 and to a lesser extend with PIP2 is crucial in order to co-localize with AKT and phosphorylate it (Anderson et al. 1998, Currie et al. 1999, Bayascas et al. 2008). This leads to partial activation, but phosphorylation of both residues is vital to generate high activity of AKT (Alessi et al. 1996, Pearce et al. 2010).

The other activating residue is at position serine 473 in AKT1 and it is located in the C-terminal hydrophobic domain. The identity of the kinase responsible for Ser- 473 phosphorylation remained elusive for long time after the Thr- 308 kinase was already discovered (Vasudevan and Garraway 2010). Now it is known that Ser- 473 is phosphorylated by a complex named mTORC2 (mammalian target of rapamycin complex 2) (Figure 8) that consists of mTOR (mammalian target of rapamycin), mLST8 (mammalian lethal with SEC13 protein 8), RICTOR (rapamycin- insensitive companion of mTOR), SIN1 (SAPK- interacting protein 1) and PROTOR (protein observed with RICTOR) (Guertin and Sabatini 2007). Phosphorylation of AKT does not happen if some of the genes encoding mTOR interacting proteins that define mTORC2 (RICTOR, mLST8 or SIN1) is deleted. This finding supports strongly the claim that AKT is directly regulated via mTORC2.

6.3. AKT substrates

6.3.1. TSC2

One of the many functions that AKT regulates (figure 9) is cell growth and protein translation through the TSC2/mTORC1 pathway (figure 10) (Vasudevan and Garraway 2010). Tuberous sclerosis 2 protein (TSC2, also known as tuberin) forms a heterodimeric complex with TSC1 (also known as hamartin) and they both together suppress mTORC1 activity (Bellacosa et al. 2004, Guertin and Sabatini 2007, Manning and Cantley 2007, Vasudevan and Garraway 2010). When TSC2 is phosphorylated by AKT, the TSC1/TSC2- complex GAP (GTPase- activating protein) activity is inhibited allowing Rheb (Ras homolog enriched in brain) binding to GTP. As a result, formed GTP-Rheb complex causes mTORC1 activation.

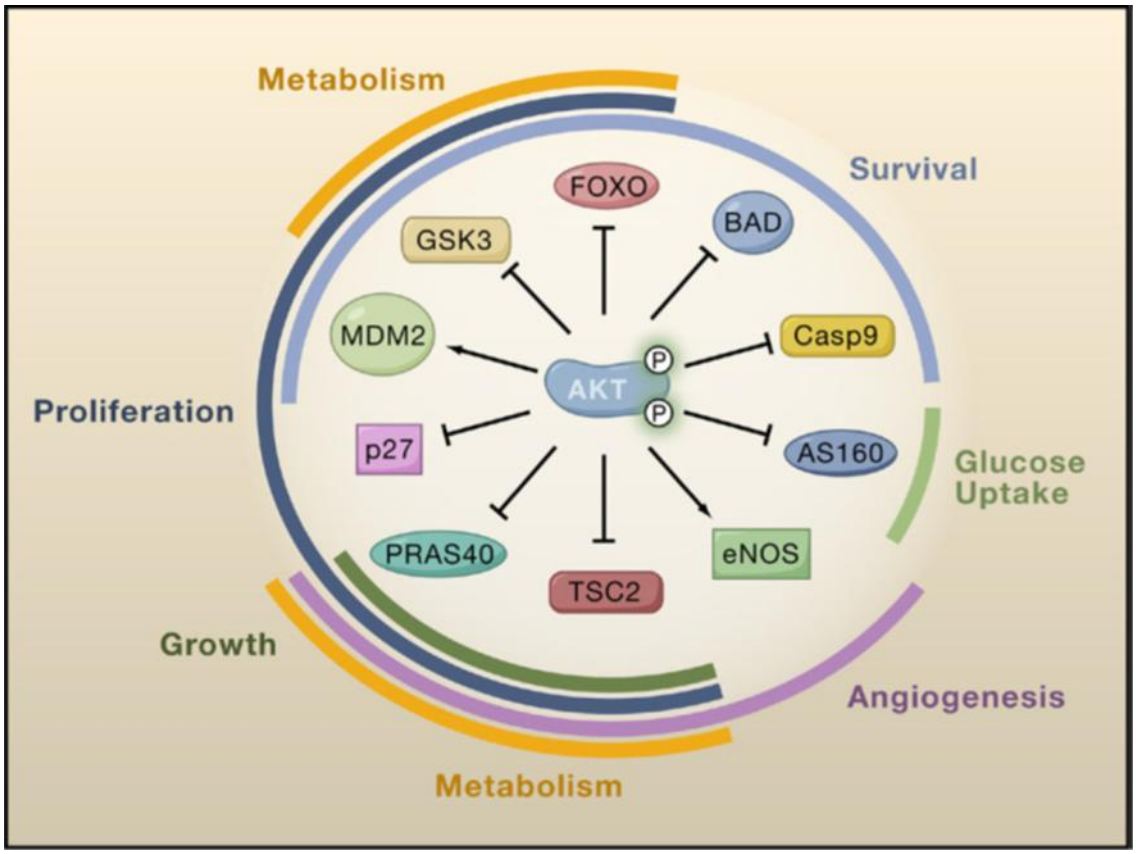


Figure 9. AKT and its many substrates (Manning and Cantley 2007).

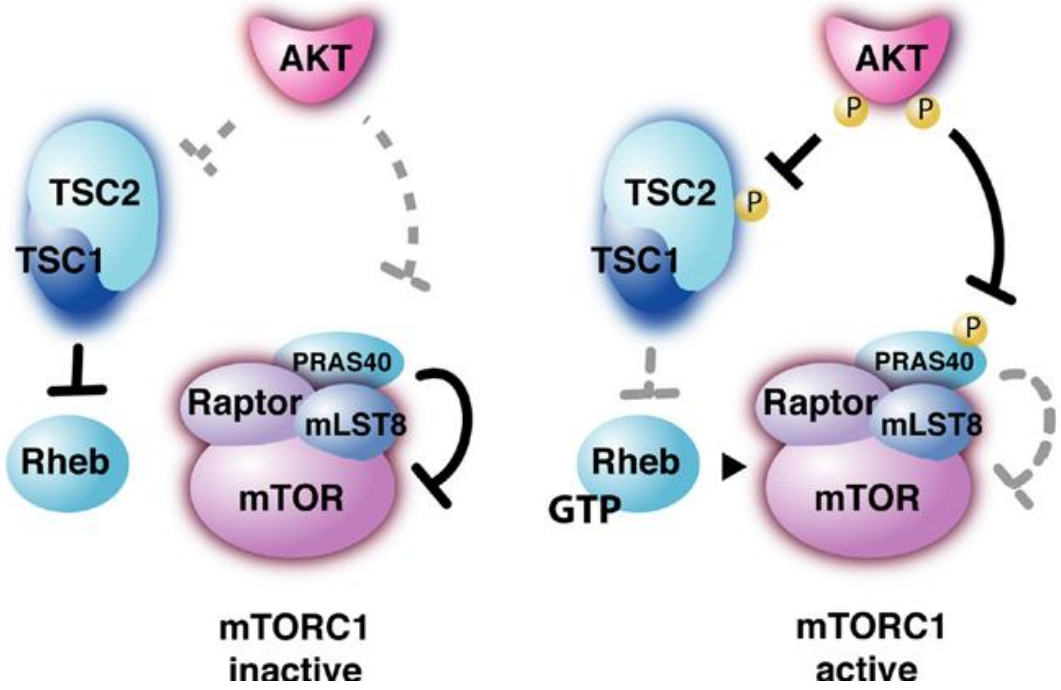


Figure 10. AKT mediated regulation of TSC2 and PRAS40 (Guertin and Sabatini 2007).

6.3.2. PRAS40

Proline-rich AKT substrate 40kDa protein (PRAS40) is phosphorylated by AKT on Thr246 (Manning and Cantley 2007, Vasudevan and Garraway 2010, McCubrey et al. 2012, Wiza et al. 2012). In the steady state PRAS40 inhibits mTORC1 activity through direct protein-protein interaction. When AKT itself becomes activated in response to insulin (figure 8), it phosphorylates PRAS40 on Thr246 causing relieve of its inhibitory action on mTORC1 (figure 10). Phosphorylation of PRAS40 enhances its binding to 14-3-3 proteins and vice versa weakens its interaction with mTORC1 enabling its activation. 14-3-3 proteins are important regulators of many client proteins by binding them and thus controlling many physiological and pathological processes (Zhao et al. 2011).

6.3.3. GSK3

Glycogen synthase kinase- 3 (GSK3) is important metabolic kinase that was found over 30 years ago (Embi et al. 1980). It was the first direct AKT substrate indentified (Cross et al. 1995). It regulates glucose and lipid metabolism (Manning and Cantley 2009) and it has role in Wnt-signalling pathway that is in essential role in embryonic development (Frame and Cohen 2001). Insulin is a GSK3 inhibitor through AKT-pathway. AKT phosphorylates GSK3 in its N- terminal serine residue and phosphorylation leads to GSK3 inactivation (Frame and Cohen 2001, Bellacosa et al. 2004, Manning and Cantley 2009).

6.3.4. FOXO

The forkhead box transcription factors (FOXO) resides in the nucleus in the absence of survival stimuli (Harris 2003, Chong et al. 2005, Manning and Cantley 2009, Vasudevan and Garraway 2010). Forkhead family mediates apoptosis, cell-cycle arrest and metabolic processes by inducing expression of many pro-apoptotic and cell- cycle inhibitory factors. Three members have been identified (FOXO1, FOXO3a and FOXO4) and all of them can be phosphorylated by AKT. Phosphorylation by AKT

leads to their export from the nucleus to the cytosol. Once in cytosol they interact with 14-3-3 proteins (Zhao et al. 2011) and their transcription of cell- death targets is blocked.

6.3.5. BAD

BAD (BCL- 2 antagonist of cell death/ BCL- 2- associated death protein) is a pro-apoptotic member of BCL- 2 (B cell CLL/lymphoma- 2) protein family (Yang et al. 1995, Adams and Cory 1998, Datta et al. 2000, Henshall et al. 2002, Chipuk et al. 2010). Family of BCL- 2 comprises of pro-survival and pro-apoptotic proteins and expression of them varies between different cells. BCL- 2 proteins play key role in controlling outer mitochondrial membrane integrity and apoptosis. Through a complex interaction between members, they regulate balance between survival and commitment to apoptosis. They have functions also in numerous cellular pathways for example in mitochondrial dynamics and endoplasmic reticulum (ER) calcium storage.

Major pro-survival members of this family (Figure 11) are: BCL- 2, long isoform (BCL-X_L), BCL- w, myeloid cell leukemia 1 (MCL- 1) and BCL- 2-related gene A1 (A1). BCL- 2 and BCL- X_L antagonize BAD so the focus will be on these two members in my thesis.

Pro-apoptotic members include proteins such as BAX (BCL-2-associated x protein), BAK (BCL-2 antagonist killer 1), BID (BCL- 2- interacting domain death agonist), BIM (BCL- 2- interacting mediator of cell death) and BAD (Figure 10& 11) (Adams and Cory 1998, Datta et al. 2000, Chipuk et al. 2010).

A conserved trait of the structure of this family members is possessing at least one of the four different conserved motifs called BCL- 2 homology domains (BH1- BH4) (Figure 11).

BAD is a distant member of this family because of its divergent structure. It contains only one of the homology domains: BH3, also known as the minimum death domain (Danial 2009). BH3- proteins are still subdivided in two classes depending on their ability to interact with other BCL- 2 family members; can they work independently or

do they bind to others and work as “sensitizators” and/or “de- repressors” (Chipuk et al. 2010).

BAD belongs to the latter group because it cannot work as an apoptotic factor independently but in order to promote apoptosis, it has to form a complex with pro-survival BCL- 2, BCL- X_L or BCL- w (Yang et al. 1995, Datta et al. 2000, Chipuk et al. 2010). In the case of BCL- X_L it is known that interaction with BAD happens through the interface formed between hydrophobic cleft of the BCL- X_L and the hydrophobic face of the amphipatic BH3 domain of BAD (Datta et al. 2000). A protective function of BCL- 2 or BCL- X_L is abolished when a tightly bound heterodimer with BAD is formed (Yang et al. 1995, Zha et al. 1996, Datta et al. 2000, Miyawaki et al. 2008).

In the absence of survival stimuli BAD works as de-repressor by replacing pro-apoptotic member BAX from heterodimer- complex with BCL- 2 or BCL- X_L and in this way causes release of BAX. Release of pro-apoptotic BAX proceeds towards mitochondrial outer membrane permeabilization, caspase activation and finally apoptosis (Yang et al. 1995, Henshall et al. 2002, Chipuk et al. 2010).

In the presence of survival stimuli BAD is at phosphorylated state and it is located in the cytosol bound with 14-3-3 protein (Zha et al. 1996, Datta et al. 2000, Zhao et al. 2011). BAD has three phosphorylation sites: Ser- 112, Ser- 136 and Ser- 155. AKT primarily phosphorylates BAD at Ser- 136 and it may have part in Ser- 112 phosphorylation as well (Datta et al. 1997). Still phosphorylation of Ser- 136 alone promotes survival by improving 14-3-3 docking (Datta et al. 2000, Danial 2009). Ser-136 phosphorylation leads to phosphorylation of Ser- 155 that is crucial in order to inhibit formation of heterodimer with BCL- X_L. Ser- 155 is located on the BH3- domain that interacts with hydrophobic groove of the pro-survival family members such as BCL- X_L. Phosphorylation of this residue makes the interaction energetically unfavorable and preventing in this way BAD to interact with BCL- X_L.

In the research of Henshall et al. 2002, it was demonstrated that AKT mediated BAD phosphorylation limited seizure-induced cell death whereas cell death was increased when AKT- pathway was blocked with PI3- kinase inhibitor (LY294002).

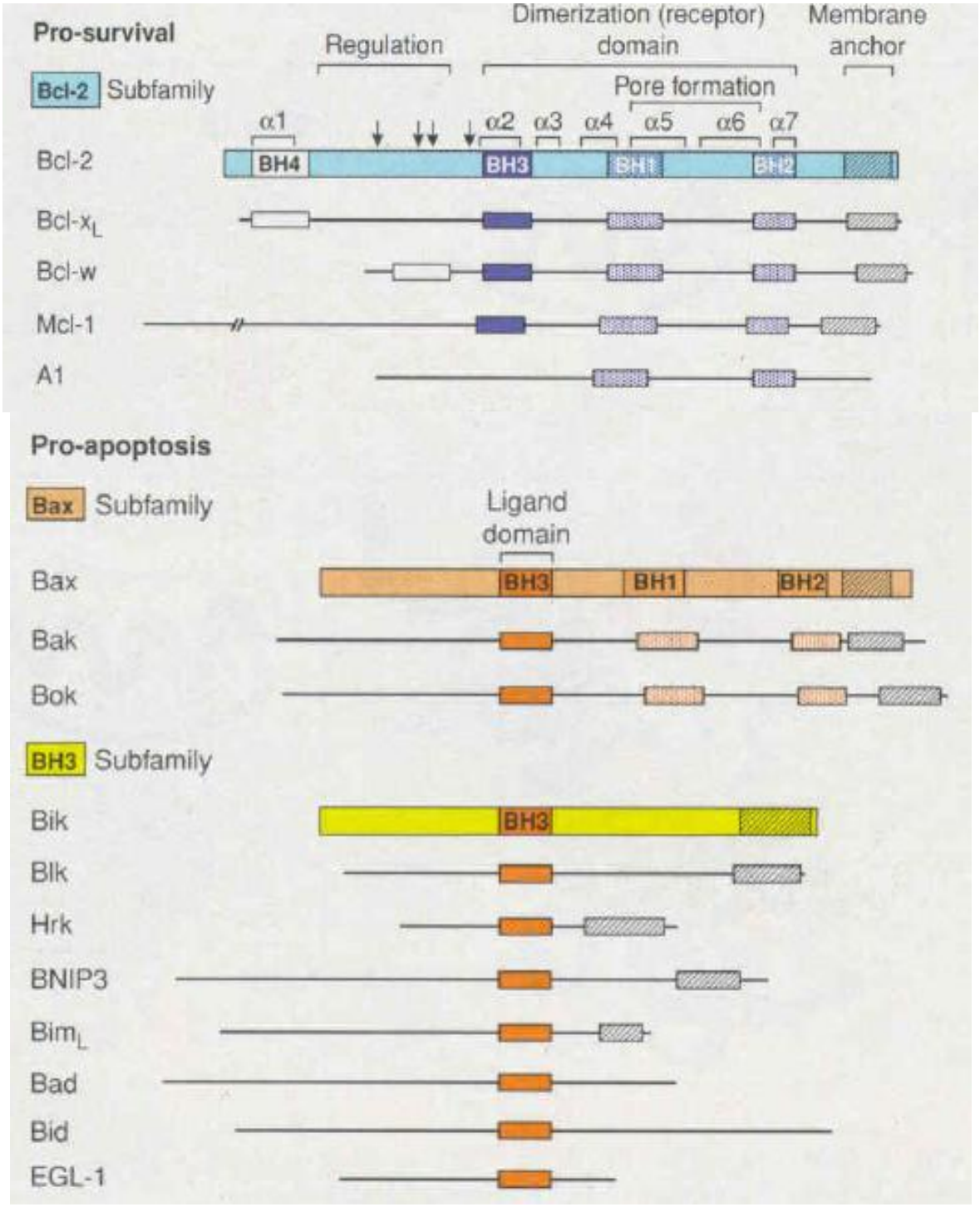


Figure 11. Basic structures of pro-apoptotic BCL-2 family members (based on BH-motifs) (Adams and Cory 1998).

6.4. PDK1 K465E- mutant mice

PDK1 K465E mutant mice were developed in the laboratory of Dario R. Alessi (Bayascas et al. 2008). It is a specific knock-in point mutation where Lysine is mutated to glutamic acid. Mutation resides in the PH domain of PDK1 that specifically binds with phosphoinositides. Mutation prevents interaction with phosphoinositides and recruitment to the plasma membrane. This affects substantially PDK1s ability to phosphorylate AKT in Thr- 308. Phosphorylation of AKT is reduced in PDK1 K465E mutant mice but not completely disappeared. Mice expressing this mutation are viable but small and insulin resistant.

6.5. Aims of the study

Aim was to build up best conditions for detecting AKT substrate BAD in its phosphorylated state, more specifically when BAD Thr- 136 is phosphorylated. This was made by altering some reagents, concentrating protein samples by using immunoprecipitation and by using phospho- specific antibodies from two different suppliers.

Another aim was to define whether Bad phosphorylation is affected or not in the PDK1 K465E neurons, and correlate that with the fact that cells survive well but do not differentiate similarly, e.g. shorter neurites of cortical neurons and aberrant axonal formation and growth in hippocampi (Zurashvili et al. 2013), as in the wild-type animals.

7. Materials and methods

7.1. Cell culture and stimulation

Cells were cortical neurons mostly from wild type mice and those were used for building up the best conditions for detection of P- BAD136(Thr). In the end of the experiment were used cortical lysates also from PDK1 K465E- mutant mice.

Cell culture

To obtain embryonic primary cultures, pregnant female mouse was sacrificed at gestational stage E15 (15^{th} day of pregnancy) counted from the appearance of the vaginal plug after mating. Both cerebral hemispheres were taken from the embryos and the rest of the body was saved for genotyping.

Cells were dissociated first enzymatically by treating them with Solution 2 (Table 2) at 37°C for 10 minutes. After 10 minutes solution 4 was added to stop the reaction. After centrifugation supernatant (mixture of Sol 2+ Sol 4) was discarded and solution 3 added on top of the pellet and cells were dissociated mechanically by gentle pipetting using a fire-polished glass Pasteur pipette. Then solution was filtrated several times through nylon mesh and cells collected and added to solution 5. Gained cell- suspension was once again centrifuged and supernatant disgarded. After this pellet was resuspended with Culture Medium I (Table 3) and cells were counted.

Table 2: Solutions used in preparation of cell culture.

Solution 1	Solution 2	Solution 3	Solution 4	Solution 5
50 ml Krebs Ringer Buffer (KRB)	10 ml Sol 1	10 ml Sol 1	84 ml Sol 1	5 ml Sol 1
0,15 mg BSA	2,5 mg trypsin	5,2 mg trypsin inhibitor	1,6 ml Sol 3	40µl $MgSO_4$ 3,8%
400µl $MgSO_4$ 3,8%		100µl $MgSO_4$ 3,8%		6 µl $CaCl_2$ 1.2%
		0,8 mg DNAse		

With the counted cell density in the solution (150 000 cells/ml), cells were plated onto poly-D-lysine (50 µg/ml) coated 24- well or 6- well plates. After 2 hour incubation medium was entirely replaced with Culture Medium II (Table 3). Cells were incubated at 37°C containing 5 % CO2 in normoxia. One half of the medium was changed for fresh medium (Culture Medium II) every 3 or 4 days.

Table 3: Culture Mediums used in a cell culture.

Culture Medium I	Culture Medium II
Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich® D-5796)	Neurobasal® (Gibco® 21103)
10% Fetal Bovine Serum (Gibco®)	B27® Serum- free supplement 50X (Gibco®, 17504)
30 mM glucose	30 mM glucose
2 mM L- glutamine	2 mM L- glutamine
0,25 mg/ml penicillin/streptomycin	0.25 mg/ml penicillin/streptomycin

Starvation and stimulation

Starvation or trophic factor deprivation was performed in order to activate apoptotic route in neurons (Zurashvili et al. 2013). It was performed at 5^{th}- 6^{th} day in vitro (DIV) by washing cells twice with serum-free DMEM and incubating them at 37°C for 4 hours with serum-free Neurobasal (supplemented with 30 mM glucose, 2 mM L-glutamine, and 0,25 mg/ml of penicillin/streptomycin).

After 4 hours of starvation cells were stimulated with BDNF (Brain-derived neurotrophic factor) diluted in DMEM. With stimulation is investigated if BDNF can recover cell viability after trophic factor deprivation (Zurashvili et al. 2013). Different exposure times were used to investigate the biochemical pathway activation. Times were 0 (control), 1, 5, 15 and 30 minutes of stimulation with BDNF. After defined time reaction was stopped by aspirating and discarding BDNF-DMEM solution and adding Lysis Buffer (Table 4) on top of the stimulated cells. Cell lysates were stored in eppendorf-tubes at -20°C.

Table 4: Content of Lysis Buffer

Tris-HCl pH 7,5	50 mM
EGTA	1 mM
EDTA	1 mM
sodium orthovanadate	1 mM
sodium fluoride	50 mM
sodium pyrophosphate	5 mM
sodium beta glicerophosphate	10 mM
sucrose	0,27 M
Triton X- 100	1% (m/V)
2-mercaptoethanol	0,1 % (V/V)
Proteinase inhibitor mixture	1% (V/V)

7.2. The Bradford assay

Method to measure quantitatively protein concentration in a solution that is based on protein binding with a dye is called Bradford assay (Boyer 2012 p.67- 69). Assay was done to all proteins used in experiments to ensure equal loading of each sample.

From each different lysate was prepared two parallel samples and their absorbance was measured with spectrophotometer. Measuring was executed immediately after adding the dye, Coomassie Brilliant Blue to the cuvette, by using wavelength 595nm. Also three blanks were measured and their average absorbance counted in order to subtract it from actual samples. Altered absorbance caused by protein binding with the dye was recorded, averages calculated and blank average absorbance subtracted. Then values were calculated as concentrations by using the standard curve prepared from bovine serum albumin (BSA).

7.3. Immunoprecipitation

Immunoprecipitation was performed in order to pull- down protein BAD from a cell lysate. Antibody that was used in the precipitation was Total- BAD mouse monoclonal antibody (table 10 primary antibodies used). The concentration was 1 or 2 µg of antibody per 10µl of A- Sepharose beads. When preparing the beads, an excess of 10µl was always added to the volume needed due to difficulties faced when pipetting the beads.

First step was washing the beads with wash buffer, centrifugation at 4°C for 1 minute, and aspiration of the buffer. This was repeated two times more. In the last aspiration approximately 50µl of wash buffer was left in the eppendorf. Nonionic detergent Triton X-100 and low salt concentration (150 mM NaCl) was used in the wash buffer (Table 5). Wash buffer was prepared by adding salt (NaCl) to Lysis buffer that was made up (content in table 4).

Table 5: Wash Buffer

Lysis Buffer	100 ml
NaCl	2,9 g

After washing the beads, antibody was pipetted into the eppendorf and it was incubated at least 30 minutes in a roller at 4°C in order to make antibody attach to the beads. During the incubation of beads with the antibody, protein samples were prepared. Amounts of proteins used for immunoprecipitation were explicitly larger than in Western Blot (see chapter 7.4.), varying between 50 up to 250µg. After 30 minutes of incubation, bead- antibody mixture was washed three times with wash buffer the same way than earlier. Then 10µl of washed bead- antibody mixture was pipetted to each tube containing proteins and they were incubated at least for 1 hour in a roller at 4°C. In this step protein desired to pull down was attached to the bead-antibody- complex.

After incubation tubes were centrifuged and supernatants were carefully pipetted to new labeled eppendorfs and saved. Pellets were washed three times with wash buffer the same way than earlier. In the last aspiration all of the liquid was removed and 40µl of wash buffer+ 10µl of loading buffer without β- mercaptoethanol was added and samples were ready to load on the polyacrylamide-gel (see chapter 7.5.1.). As a control was used bead- antibody mixture in a wash buffer. This was made to ensure that antibody is attached to the beads (that beads are working correctly) and no free antibody remains in the wash buffer.

7.4. Preparing the samples for Western Blot

By using calculated concentrations (see chapter 7.2.), volumes of protein lysates needed for loading were calculated so that the amount of loaded protein were equal in each well. If needed, water was added to gain wanted volume. Amounts per loading varied between 10- 40µl (amounts of proteins varied from 12,5µg up to 60µg) in separate Western Blots due to shortage of lysates. To each sample was added loading buffer with ratio 1:5 that contained bromophenol blue and β-mercaptoethanol in order to monitor the progress of electrophoresis and to break the disulfide bonds,

respectively. As mentioned in the previous chapter 7.3., to the proteins that were immunoprecipitated with anti- BAD β- mercaptoethanol was not added because of its ability to denature the antibody and distract immunoprecipitation (Simpson et al. 2009).

To break the three dimensional structure of a protein, surrounding conditions for the protein has to be made unfavorable (Campbell and Farrell 2006 p.99- 101). It is also called denaturing and it can be done by heating, altering pH or with detergents. Methods that was used in this experiment was added sodium dodecyl sulfate (SDS) to the polyacrylamide gel (Berg et al.2002 p.84, Campbell and Farrell 2006 p.101) and by heating samples just before loading.

7.5. Western blot

When it is necessary to detect small amount of a certain protein from a mixture of many proteins (e.g. cell lysate), immunoassay technique called Western Blot is a good procedure to use (Berg et al. 2002 p.103, Lodish et al. 2008 p.98, Boyer 2012 p.194). It is a multistep procedure, a combination of techniques that makes possible to separate proteins and detect and observe a protein of interest (Lodish et al. 2008 p.98). It is a similar technique as Southern Blotting but instead of transferring DNA, Western Blot is designed to work with proteins (Berg et al. 2002 p.146, Campbell and Farrel 2006 p.358). The procedure consists of SDS- polyacrylamide- gel electrophoresis (SDS- PAGE), electroblotting and antibody-antigen based detection of proteins of interest (Berg et al. 2002 p.103, Campbell and Farrel 2006 p.121, Boyer 2012 p.194- 195).

7.5.1. SDS- PAGE

In order to fractionate proteins, they are forced to migrate through polyacrylamide gel by electric field (Berg et al. 2002 p.84, Boyer 2012 p.194). Prefix SDS (sodium dodecyl sulfate) stand for denaturing conditions (see chapter 7.4.). Movement of proteins is based on their molecular sizes and it determines their separation. At the

end of the electrophoresis small proteins are at the bottom, whereas large proteins stay near to the place of loading (Berg et al. 2002 p.84-85, Campbell and Farrel 2006 p.121).

Used concentrations of gels in this experiment were either 7,5% or 12%, where concentration of 12% provided bigger resistance for proteins. Gels were prepared according to the table 6, in such a way that the running gel was at the bottom and stacking gel including the loading wells were on top of that. Equal amount of lysate varying between 10µl- 40µl (in separate gels) were loaded in the wells and fractionated with electric current of 160V approximately for one and a half hour (depending of the density of the gel; 7,5% gel completed in lesser time). To be able to identify the different molecular weights afterwards, protein marker Precision Plus Protein™ Standard (Bio Rad Laboratories, Inc) was loaded also in the acrylamide, usually in the outermost well/ wells. Electrophoresis happened in a buffer, which content is shown in table 7.

Table 6: Gels for Western Blot

For 2 gels	**Stacking**	**Running**	
SOLUTION		**7,50 %**	**12 %**
30% Acrylamide/ 0,8% Bis solution (37,5:1) (Merck, Germany)	850 µL	5 mL	8 mL
H_20 (distilled)	3,8 mL	10 mL	7 mL
1,5 M Tris- Base (pH 8,8) (Sigma, USA)	-----	5,0 mL	5,0 mL
0,5 M Tris- Base (pH 6,8) (Sigma-USA)	325 µL	---	---
20 % SDS (Sigma, Japan)	25 µL	0,25 mL	0,25 mL
10 % APS (Sigma, USA)	50 µL	150 µL	150 µL
TEMED (Sigma, China)	5 µL	30 µL	30 µL

Table 7: SDS- electrophoresis buffer

Tris-Base (Sigma, USA)	3,0 g
Glycine (Sigma, South Korea)	14,4 g
SDS (Sigma, Japan)	1,0 g
H_2O (distilled)	ad 1,0 l

7.5.2. Electroblotting

After proteins are fractionated by electrophoresis they are still deeply embedded in the polyacrylamide gel (Berg et al. 2002 p.84, Campbell and Farrel 2006 p.358, Lodish et al. 2008 p.98, Boyer 2012 p.194). In order to get proteins more accessible they have to be transferred (blotted) to a support matrix that allows working with strongly bound and immobilized proteins. Different types of support matrixes exist, but in this experiment was used nitrocellulose membrane.

For the transfer a cassette is assembled according to the figure 12 so that the electric current transfers proteins from the polyacrylamide gel onto the membrane. Order in the cassette is sponge, filter paper x2, gel, membrane, filter paper x2 and sponge. Casette is placed between platinum electrodes and electric current is then passed through the gel and proteins electrophorese from the gel to the membrane. Like electrophoresis, electroblotting happens also in a buffer solution (table 8), at 100V for 90 minutes and temperature lowered near 4°C with ice.

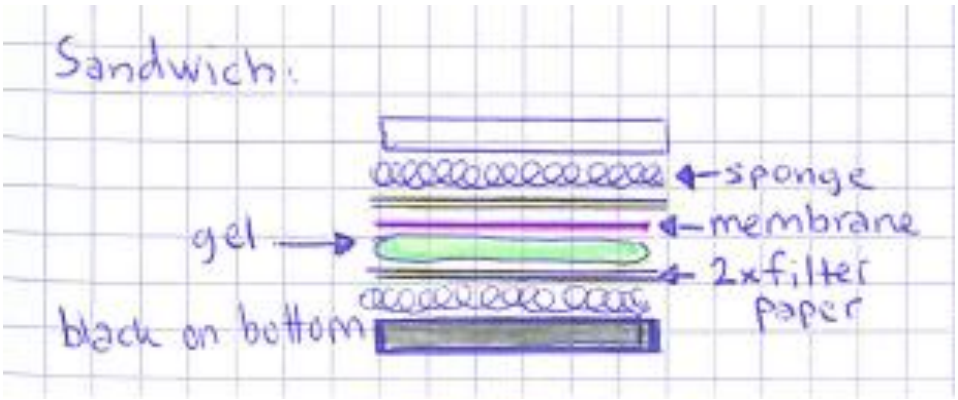


Figure 12. Arrangement in the transfer cassette (sandwich).

Table 8. Transfer (blotting) buffer

Tris- Base (Sigma, USA)	3,0 g
Glycine (Sigma, South Korea)	15,0 g
Methanol	200 ml
H_2O (distilled)	ad 1,0 l
After preparation cooled in a freezer to mitigate produced heat during the transfer.	

7.5.3. Antibody-antigen based detection

Immunodetection includes following steps: blocking the membrane, incubation with primary antibody, incubation with secondary antibody, adding chemiluminescent substrate and exposure and develop of a film (Berg et al. 2002 p.103, Lodish et al. 2008 p.98, Boyer 2012 p.195-197).

Blocking the membrane with protein solution ensures that the primary antibody doesn't interfere with other than blotted proteins. In this research was used either casein (major protein in milk) or BSA (bovine serum albumin) in TBS-Tween. Blocking took place at room temperature for 45 minutes.

After blocking, the membrane was incubated with specific primary antibody that binds to desired proteins. Used concentration of primary antibody was usually 1:1000, but in some cases higher concentrations were used; 1:500, 1:200 or even 1:100. Primary antibodies were diluted in 0,5% or 5% BSA depending whether antibody was phospho or total, respectively. Incubation was performed at 4°C usually for 12- 18 hours or more. After washing the membrane from excess of primary antibody (3x 10 minutes with TBS- Tween) the secondary antibody (Table 11) conjugated with HRP (horseradish peroxidase) was directed against the primary antibody to interact with it. This took place at room temperature for 50 minutes. Used concentrations with secondary antibody were 1:2500 in 5% milk. Washes were repeated to remove excess of secondary antibody as well. All incubations and washes were performed in a horizontal roller.

Enzyme that is conjugated with secondary antibody (HRP) catalyzes the reaction that produces light signal. Composition of mixture that was used to produce chemiluminescent is seen in table 9. After mixing ECL1 and 2 together, the reaction start to happen immediately so the incubation with the membrane had to be short (was 1 minute) not to lose adequate light signal. Right after this film (Fuji Film Super RX) was exposed to light producing membranes in the darkroom and then developed.

Table 9: Composition of ECL1 and 2 (enhanced chemiluminescence). Mixed together just before incubating the membranes.

ECL 1	
1 M Tris HCl (pH 8,5)	200 µl
0,5M Luminol	10 µl
79.2 mM p-Coumaric Acid	10 µl
H_2O (distilled)	ad 2 ml

ECL 2	
1 M Tris HCl pH 8,5	200 µl
8.8 M Hydrogen Peroxide (H_2O_2)	1,3 µl
H_2O (distilled)	ad 2 ml

Table 10: Primary antibodies used

Antibody	Host	Provider	Cataloque number
P-BAD S112	Rabbit	Cell Signaling Technology	5284
P-BAD S136	Rabbit	Cell Signaling Technology	5286
P-BAD S155	Rabbit	Cell Signaling Technology	9297
Total BAD	Rabbit	Cell Signaling Technology	9239
P-BAD S136	Rabbit	Cell Signaling Technology	9295S
Total BAD	Mouse	BS Biosciences	36919
P-AKT T308	Rabbit	Cell Signaling Technology	9275
P-AKT S473	Rabbit	Cell Signaling Technology	9271
Total AKT	Sheep	Dario R. Alessi/ University of Dundee	

Table 11: Secondary antibodies used

Antibody	Host	Provider	Cataloque number
Anti-Rabbit HRP-conjugated	Goat	Thermo Scientific	31460
Anti-Mouse HRP-conjugated	Goat	Thermo Scientific	31430
Anti-Sheep HRP-conjugated	Rabbit	Thermo Scientific	31480

8. Results and discussion

8.1. Effect of immunoprecipitation

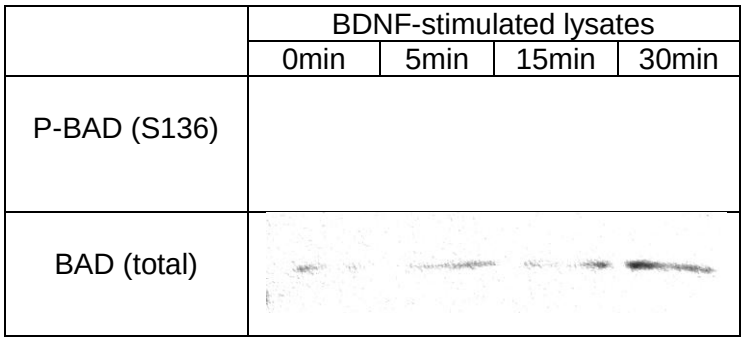


Figure 13. Detecting P-BAD(S136) and BAD(total) from cortical cell lysates from wild type mice stimulated with BDNF at different timepoints.

On one of the first experiments was examined if BAD could be detected from regular cortical cell lysates (from wild type mice). As can be seen in figure 13 P- BAD (S136) cannot be detected but BAD (total) can, although weakly. From this result was concluded that protein concentration has to be higher (e.g. 50- 250µg instead of normal loading amount of 10- 25µg) to be able to detect P-BAD(S136) and also get stronger and more reliable signal from BAD(total).

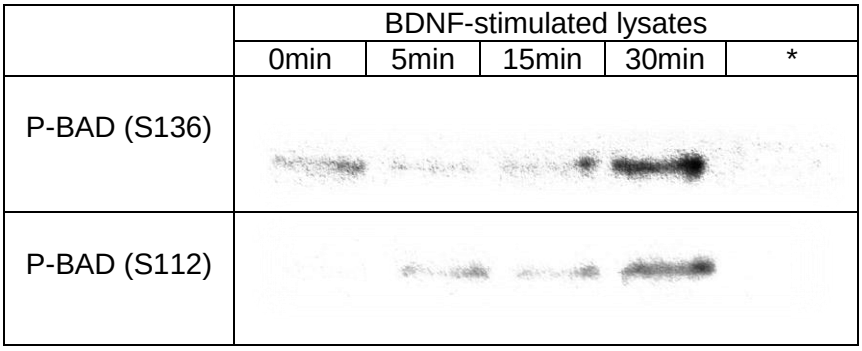


Figure 14. Detection of phosphorylated BAD at residues S136 and S112 in immunoprecipitated BDNF-stimulated cortical cell lysates. Control is marked as *, which consist of bead-antibody complex in a wash buffer.

After lysates were concentrated by immunoprecipitation, detection of P-BAD136 and P-BAD112 succeeded (figure 14). This finding supported conclusion that cortical cell lysates has to be concentrated in order to detect BAD and especially its phosphorylated states and it is recommended that at least 15 min incubation will be used to activate the process by BDNF.

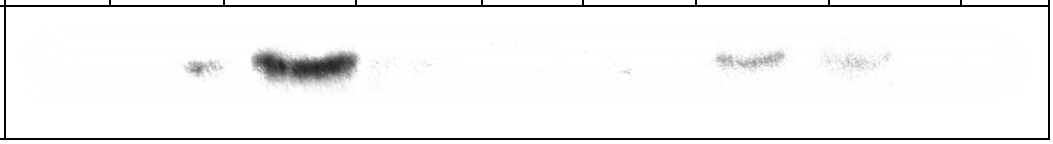
	Total lysates				Supernatant				
	BDNF-stimulation time								
	0min	5min	15min	30min	0min	5min	15min	30min	*
BAD (total)									

Figure 15. Saved supernatant was used in the experiment in order to detect how well immunoprecipitation succeeded (see chapter 7.3.).

Figure 15 show difference of BAD(total) detection between immunoprecipitated (concentrated) lysates and supernatant gained from immunoprecipitation. Some protein is still left with samples in timepoints 15 and 30 min. Especially sample in timepoint 15 min correlates with weaker detection of P-BAD (S136) and P-BAD (S112) in timepoint 15 min in figure 14.

	Total lysates				Supernatant				
	BDNF-stimulation time								
	0min	5min	15min	30min	0min	5min	15min	30min	*
AKT (total)									

Figure 16. Detection of AKT(total).

Figure 16 represents AKT as a control of loading. Results are from the same Western blot than results in figure 15, although this is the upper part of the same membrane. Detection should be equal in each timepoint. From the figure can be concluded that the loading is a bit uneven.

	Total lysates				Supernatant				
	BDNF-stimulation time								
	0min	5min	15min	30min	0min	5min	15min	30min	*
BAD (total)									

Figure 17: Another experiment presenting the difference of BAD(total) detection between immunoprecipitated (concentrated) lysates and supernatant gained from immunoprecipitation where most of the protein is gone because of the attachment to the BAD- antibody- bead- complex.

As can be seen from the figure 17 immunoprecipitation succeeded better with samples at timepoints 0 and 5 minutes than in 15 and 30 minutes. Some protein is still left in the supernatant at timepoints 15 and 30 minutes.

	Total lysates				Supernatant				
	BDNF-stimulation time								
	0min	5min	15min	30min	0min	5min	15min	30min	*
AKT (total)									

Figure 18: Upper part of the same membrane which was used to detect BAD in Figure 17, was treated with AKT(total) antibody to get control of loading.

Figure 18 shows that detection of AKT(total) is also a bit weaker in supernatant at timepoints 0 and 5 minutes than in total lysate samples. This should not happen because immunoprecipitation with BAD(total)- antibody cannot affect to the amount of AKT. This weaker signal can be explained by inaccurate loading of samples.

However, loading is more accurate than in figure 16, especially with loading of total lysates.

8.2. Antibodies from different suppliers

	BDNF-stimulated lysates			
	0min	5min	15min	30min
P-BAD (S112) (5284)				
P-BAD (S136) (5286)				
BAD (total) (9239)				
P-AKT (S473)				

Figure 19: Detection with antibodies from Cell Signalling Technology. P- AKT(S473) as a control.

P-BAD S112 (5284), P-BAD S136 (5286) and Total BAD (9239) antibodies from Cell Signalling Technology (table 10 on page 51) were tested with cortical cell lysates from wild type mouse without immunoprecipitation. P-AKT (S473) was used as a control. In the figure 19 cannot be seen signals from P-BAD (S112), P-BAD (S136) or BAD (total). Some signals were detected, but molecular weight did not match to the BAD (23kDa). However, signal produced by P-AKT (S473) is strong and it demonstrates that phosphorylation of AKT is not impaired in these neurons.

Based on the experiments was discovered that the ability of Total BAD #9239 (Cell Signalling Technology) to pull- down BAD from lysates in immunoprecipitation was weaker than with Total BAD (BS Bioscience) (results not shown). Because of this

observation all later immunoprecipitations were performed with Total BAD (BS Bioscience).

It was also found that P-BAD (S136) #9295S (Cell Signalling Technology) works better than P-BAD (S136) #5286 (Cell Signalling Technology). P-BAD (S136) #5286 was also tested with concentrated lysates (results not shown) but no detection was observed with this specific anti- body. In all later experiments was used #9295S to detect phosphorylation of BAD(S136).

8.3. PDK1 K465E neurons

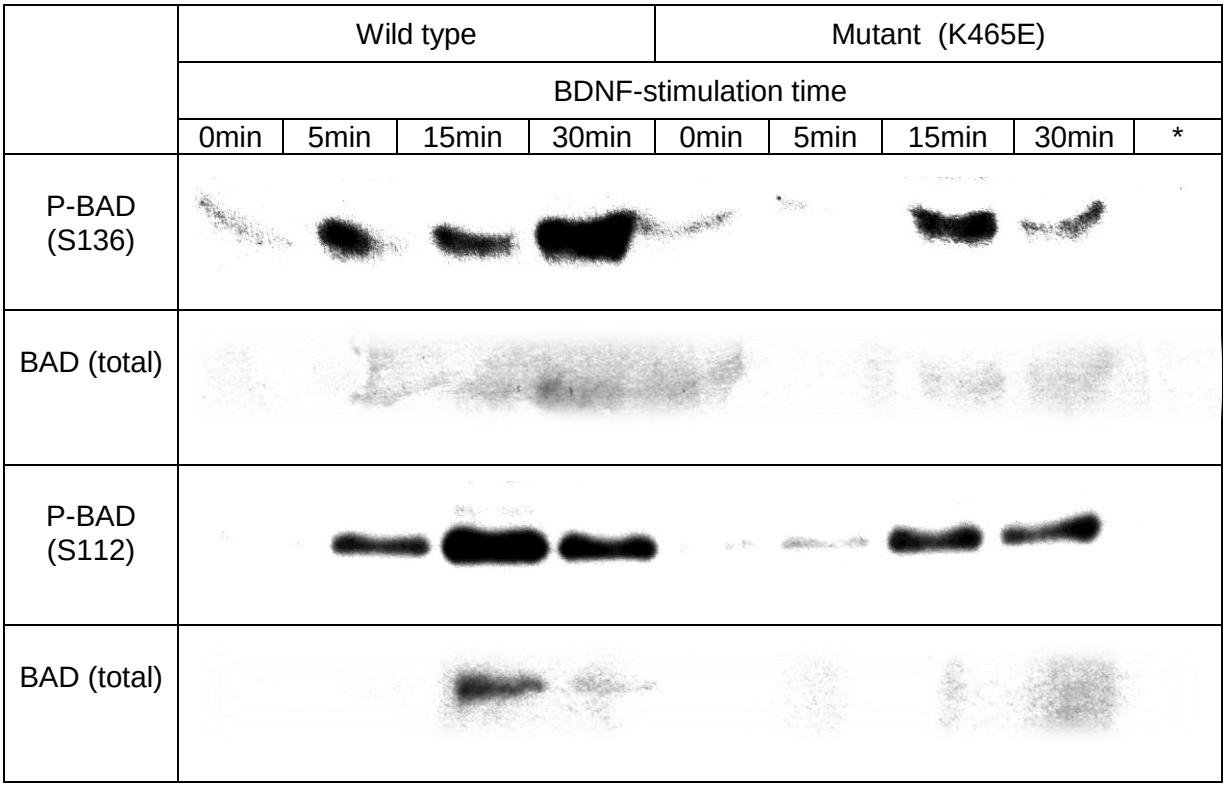


Figure 20. Phosphorylation of BAD residues 136 and 112, and same membranes treated afterwards with total-BAD antibody.

Cortical cell lysates gained from wild type and mutant mice were first immunoprecipitated according to the earlier findings that lysates has to be concentrated in order to detect P-BAD 136 and 112. As figure 20 shows detection of

phosphorylation succeeded. Even though totals barely can be seen (film got wet) some signal can be detected and that supports reliability of P- BAD(112 and 136) signals.

It seems that phosphorylation of S112 and S136 is weaker in mutant lysates compared to wild type. With both lysates phosphorylation of S112 seems to be strongest at timepoint 15 min. Detection of S136 is strongest at 30 min with wild type and at 15 min with mutant. Possibly 15 minute exposure in BDNF is optimal time after starvation to activate the biochemical pathway in mutant lysates.

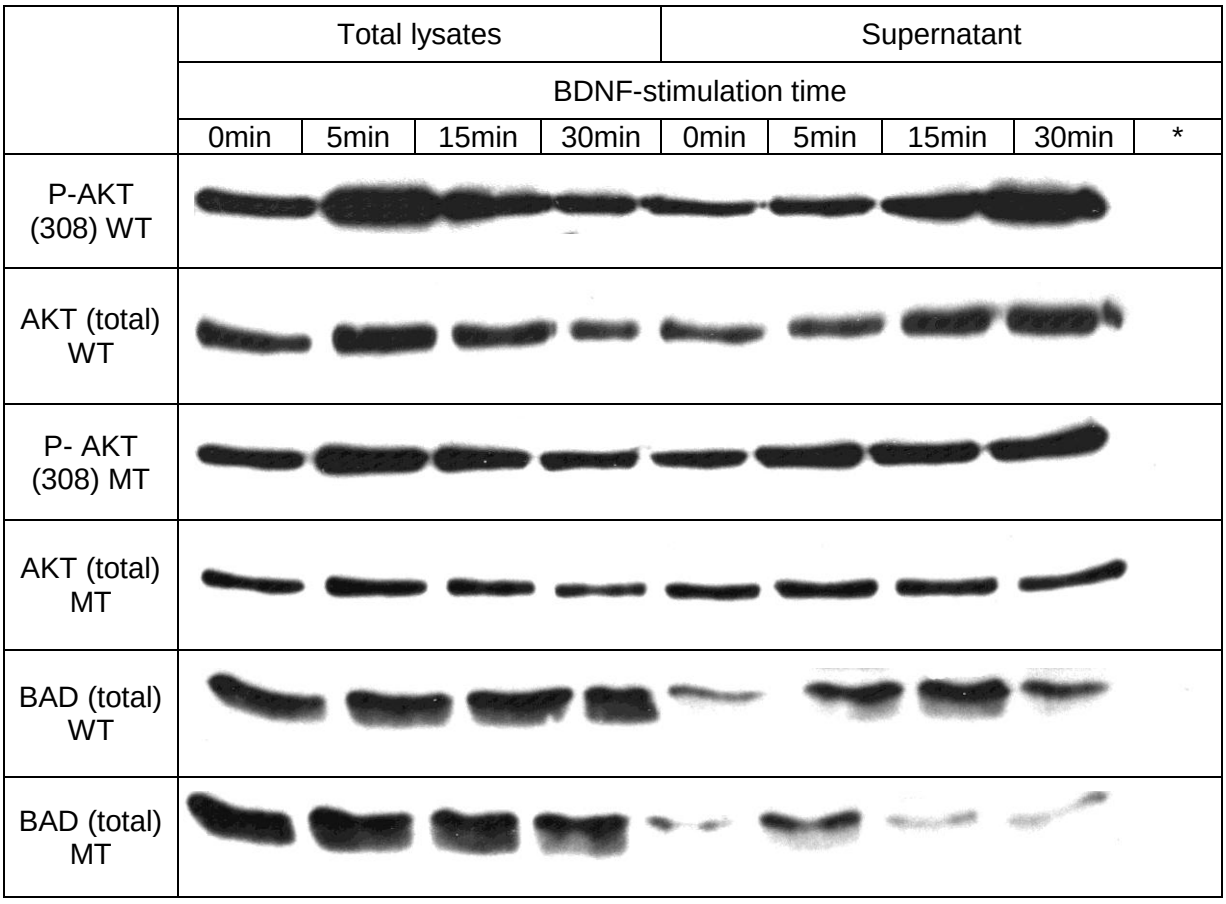


Figure 21. Loading controls (AKTs) and results of succeeding of the immunoprecipitation.

As can be seen from the figure 21 (based on AKT(total)s) loadings are quite even. Some wells have bigger amount of lysate and that correlates with stronger detection of corresponding P- AKT(308). When comparing detection of phosphorylated

AKT(308)s to the corresponding AKT(total)s, it seems that detection is a bit stronger in WT than in MT lysates.

When observing immunoprecipitated lysates, some amount of protein is still left in the supernatant. With WT in timepoints 5, 15 and 30 min signal is especially strong. When comparing these timepoints to the figure 20 it does not seem likely that immunoprecipitation failed because signals are so strong. Explanation may be the saturation of beads.

With MT in timepoint 5 min some protein is left in supernatant (figure 21) and it correlates with weaker signal of P- BAD(S136) and P- BAD(S112) (figure 20). Despite this, can be concluded that immunoprecipitation succeeded quite well because lysate seems to be rich of protein.

9. Conclusions

Even though the lysates were immunoprecipitated, protein was hard to detect and signals appeared faintly. Larger amount of antibody was used when concentrations had to be elevated in order to detect P-BAD. This kind of research requires large amount of resources. Generating specific mouse genotype and establishing embryonic primary cultures requires a lot of time and effort. Also as a method, Western Blot is a bit inaccurate because it was based only on a visual estimate result from a lack of equipment.

It was also observed that as a protein, BAD is very small and hard to detect especially on its phosphorylated state. Concentration of protein was very low at cortical lysates and it was noticed that protein degrade easily when adding β-mercaptoethanol. From these observations can be concluded that BAD is a delicate protein to work with and it requires plenty of skills and precision from the worker.

Because of the lack of time and shortage of PDK1 K465E- lysates, only two parallel experiments were performed with mutated neurons. Hence, more experiments have to be conducted to get more reliable data.

Scientific value is also affected by the precision of the worker. Results indicate that some stages would have required more precision, for example there are variations in loadings. Again, more repeats would reduce this defect.

In the base of this research it seems that PDK1 K465E- mutation has effect in phosphorylation of pro- apoptotic BAD. Phosphorylation of BAD Ser- 136 was weaker in mutant than in wild type. This indicates that when AKT activation is impaired in consequence of its hypophosphorylation, it decreases also phosphorylation of BAD at Ser-136. This would mean that BAD attaches to BCL- 2 or BCL- X_L and in this way causes release of pro- apoptotic BAX that further leads to apoptosis.

In the research of Rokhlin et al. (2004) was investigated the effect of inhibition of PDK1/AKT pathway on phosphorylation of BAD Ser- 136 in prostatic carcinoma cell lines. Results indicated that when PDK1/AKT pathway activation was impaired, phosphorylation of BAD Ser- 136 was subsequently reduced. Although inhibition lowered phosphorylation of PDK1-Ser241 and did affect in PH-domain in no manner,

it supports the fact that PDK1 has to be fully activated in order to phosphorylate AKT-Thr308 and subsequently AKT to phosphorylate BAD Ser-136. This strongly supports the importance of PDK1/AKT pathway on inhibiting apoptotic cascade via phosphorylation of pro-apoptotic BAD.

Phosphorylation of BAD Ser- 136 is known to improve docking with 14-3-3 proteins and as well promote phosphorylation of Ser- 155 which is important step to inhibit formation of heterodimer with BCL- X_L. If phosphorylation is completely impaired, it would be improbable that mice would be viable in birth.

Previous data from experiments done with neurons from PDK1 K465E- mutant mice indicated that mice were viable, only smaller and insulin resistant (Zurashvili 2013). Also phosphorylation and inhibition of other apoptotic substrates of AKT, such as GSK3 or FOXO were shown to be normal and their inhibition was not decreased.

It is possible that even if AKT function is impaired, it can still phosphorylate BAD at Ser-136 at some level or BAD is phosphorylated by some other kinase. If BAD phosphorylation indeed is impaired in PDK1 K465E- neurons could that be the causative factor to the small size of the mice if other substrates are phosphorylated normally? Further investigation is needed with PDK1 K465E-neurons to demonstrate whether BAD Ser- 136 phosphorylation is affected or not.

10. References

Adams JM, Cory S: The BCL-2 protein family; arbiters of cell survival. Science 281:1322-1326, 1998

Alessi DR, Andjelkovic M, Caudwell B et al.: Mechanism of activation of protein kinase B by insulin and IGF-1. The EMBO Journal 15(23):6541- 6551, 1996

Anderson KE, Coadwell J, Stephens LR, Hawkins PT: Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. Current Biology 8:684– 691,1998

Apostolova LG, Green AE, Babakchanian S, Hwang KS, Chou Y-Y, Toga AW, Thompson PM: Hippocampal atrophy and ventricular enlargement in normal aging, mild cognitive impairment and Alzheimer's disease. Alzheimer disease and associated disorders 26(1):17- 27, 2012

Barron AM, Pike CJ: Sex hormones, aging, and Alzheimer's disease. Frontiers in bioscience (Elite Edition) 4:976- 997, 2012

Bayascas JR, Wullschleger S, Sakamoto K et al.: Mutation of the PDK1 PH Domain Inhibits Protein Kinase B/AKT, Leading to Small Size and Insulin Resistance. Molecular Cell Biology 28(10): 3258- 3272, 2008

Bellacosa A, Testa JR, Moore R, Larue L: A portrait of AKT kinases: human cancer and animal models depict a family with strong individualities. Cancer Biol Ther 3:268-275, 2004

Berg JM, Tymoczko JL, Stryer L: Biochemistry. 5th edition, W.H. Freeman and Company, New York USA, 2002

Boyer R: Biochemistry laboratory: Modern theory and techniques. 2nd edition, Pearson Education, Inc., New Jersey USA, 2012

Campbell MK, Farrel SO: Biochemistry. 5th edition, Thomson Brooks/ Cole, Belmont USA, 2006

Carroll JC, Rosario ER: The potential use of hormone-based therapeutics for the treatment of Alzheimer's disease. Current Alzheimer Research 9:18- 34, 2012

Casamayor A, Morrice NA, Alessi DR: Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo. The Biochemical Journal 342:287- 292, 1999

Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR: The BCL-2 Family Reunion. Molecular Cell 37(3):299- 310, 2010

Chong ZZ, Li F, Maiese K: Activating Akt and the brain's resources to drive cellular survival and prevent inflammatory injury. Histology and histopathology, 20(1): 299-315, 2005

Chu LW: Alzheimer's disease: early diagnosis and treatment. Hong Kong medical journal 18(3):228- 237, 2012

Chudek JA, van Diepen JA, Voshol PJ, Lucocq JM, and Alessi DR: Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance. Molecular Cell Biology 28(10):3258- 3272, 2009

Correia SC, Santos RX, Perry G: Mitochondrial Importance in Alzheimer's, Huntington's and Parkinson's Diseases. In a book: Neurodegenerative Diseases, vol. 724, p.205-221. Edited by Ahmad SI , Landes Bioscience and Springer Science+Business Media, LLC, New York USA 2012

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA: Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*: 785- 789, 1995

Currie RA, Walker KS, Gray A et al.: Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1. The Biochemical Journal *337*: 575- 583, 1999

Danial NN: BAD: undertaker by night, candyman by day. Oncogene 27:S53- S70, 2009

Datta SR, Dudek H, Tao X et al.: Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91(2):231- 241, 1997

Datta SR, Katsov A, Hu L et al.: 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Molecular Cell 6(1):41- 51, 2000

Duan Y, Dong S, Gu F, Hu Y, Zhao Z: Advances in pathogenesis of Alzheimer's disease: focusing on tau-mediated neurodegeneration. Translational neurodegeneration 1(1):24, 2012

Embi N, Rylatt DB, Cohen P: Glycogen Synthase Kinase-3 from Rabbit Skeletal Muscle. European journal of biochemistry 107: 519- 527,1980

Farlow M: A Clinical Overview of Cholinesterase Inhibitors in Alzheimer's Disease. International Psychogeriatrics 14(1): 93- 126, 2002

Frame S, Cohen P: GSK3 takes centre stage more than 20 years after its discovery The Biochemical Journal 359:1- 16, 2001

Färkkilä M, Pirttilä T: Muut neurologiset sairaudet. In a book: Kliininen farmakologia ja lääkehoito. 2.edition, p.571- 574. Edited by Neuvonen PJ, Backman JT, Himberg J-J, Huupponen R, Keränen T, Kivistö KT, Kandidaattikustannus Oy, Helsinki 2011

Gauthier S, Wu L, Rosa-Neto P, Jia J: Prevention strategies for Alzheimer's disease. Translational neurodegeneration 1(1):13, 2012

Ghosh AK, Brindisi M, Tang J: Developing β-secretase inhibitors for treatment of Alzheimer's disease. Journal of Neurochemistry 120(1): 71– 83, 2012

Guertin DA, Sabatini DM: Defining the Role of mTOR in Cancer. Cancer Cell 12:9- 22, 2007

Götz J, Chen F, van Dorpe J, Nitsch RM: Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Abeta 42 fibrils. Science 293(5534):1491- 1495, 2001

Hanada M, Feng J, Hemmings BA: Structure, regulation and function of PKB/AKT- a major therapeutic target. Biochimica et Biophysica Acta 1697:3- 16, 2004

Hardy J, Selkoe DJ: The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics. Science 297(5580):353- 356, 2002

Harris TK: PDK1 and PKB/Akt: ideal targets for development of new strategies to structure-based drug design. IUBMB life 55(3):117- 126, 2003

Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB: Exploiting the PI3K/AKT pathway for cancer drug discovery. Nature reviews drug dicovery 4:988- 1004, 2005

Henshall DC, Araki T, Schindler CK et al.: Activation of Bcl-2-associated death protein and counter-response of Akt within cell populations during seizure-induced neuronal death. The journal of neurosciences 22(19):8458- 8465, 2002

Hers I, Vincent EE, Tavaré JM: Akt signalling in health and disease. Cellular Signalling 23: 1515– 1527, 2011

Hickman SE, Allison EK, El Khoury J: Microglial dusfunction and defective β- amyloid clearance pathways in aging Alzheimer's disease mice. Neurobiology of disease 28(33):8354- 8360, 2008

Hong-Qi Y, Zhi-Kun S, Sheng-Di C: Current advances in the treatment of Alzheimer's disease: focused on considerations targeting Aβ and tau. Translational Neurodegeneration 1(1):1-21, 2012

Huang Y, Mucke L: Alzheimer mechanisms and therapeutic strategies. Cell 148(6):1204- 1222, 2012

Kimberly WT, Xia W, Rahmati T, Wolfe MS, Selkoe DJ: The transmembrane aspartates in presenilin 1 and 2 are obligatory for γ-secretase activity and amyloid β-protein generation. The journal of biological chemistry 275(5):3173- 3178, 2000

Knopman D: Alzheimer's disease. In a book: Principles and practice of geriatric psychiatry. p. 283- 300. Editors Agronin ME, Maletta GJ, Lippincott Williams& Wilkins, Philadelphia USA 2006

Lawlor MA, Alessi DR: PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? Journal of Cell Science 114: 2903- 2910, 2001

Lippa CF, Saunders AM, Smith TW, et al.: Familial and sporadic Alzheimer's disease: neuropathology cannot exclude a final common pathway. Neurology 46(2):406- 412, 1996

Lodish H, Berk A, Kaiser CA et al.: Molecular cell biology. 6th edition, W.H. Freeman and Company, New York USA, 2008

Manning BD, Cantley LC: AKT/PKB Signaling: Navigating downstream. Cell 129(7):1261-1274, 2007

McCubrey JA, Steelman LS, Chappell WH et al.: Mutations and Deregulation of Ras/Raf/MEK/ERK and PI3K/PTEN/AKT/mTOR Cascades. Oncotarget 3(9):954-987, 2012

Miyawaki T, Mashiko T, Ofengeim D et al.: Ischemic preconditioning blocks BAD translocation, Bcl-x_L cleavage, and large channel activity in mitochondria of postischemic hippocampal neurons. Proceedings of the National Academy of Sciences of the United States of America 105(12):4892- 4897, 2008

Newman EL, Gupta K, Climer JR, Monaghan C, Hasselmo ME: Cholinergic modulation of cognitive processing: insights drawn from computational models. Frontiers in behavioral neuroscience 6:24, 2012

Parker PJ, Parkinson SJ: AGC protein kinases phosphorylation and protein kinase C. Biochem. Soc. Trans 29:860- 863, 2001

Parthsarathy V, McClean PL, Hölscher C, et al.: A novel retro- inverso peptide inhibitor reduces amyloid deposition, oxidation and inflammation and stimulates neurogenesis in the APPswe/ PS1ΔE9 mouse model of Alzheimer's disease. PLoS one 8(1):e54769, 2013

Pearce LR, Komander D, Alessi DR: The nuts and bolts of AGC protein kinases. Nature reviews. Molecular cell biology 11(1):9- 22, 2010

Placanica L, Tarassishin L, Yang G, Peethumnongsin E, Kim SH, Zheng H, Sisodia SS, Li YM: Pen2 and presenilin-1 modulate the dynamic equilibrium of presenilin-1 and presenilin-2 gamma-secretase complexes. The journal of biological chemistry 284(5):2967- 2977, 2009

Povova J, Ambroz P, Bar M, Pavukova V, Sery O, Tomaskova H, Janout V: Epidemiological of and risk factors for Alzheimer’s disease: A review. Biomedical papers of the Medical Faculty of the University of Palacky, Olomouc, Czechoslovakia 156(2):108-114, 2012

Querfurth HW, LaFerla FM: Alzheimer's disease. The New England journal of medicine 362(4):329- 44, 2010

Rapoport M, Dawson HN, Binder LI, Vitek MP, Ferreira A: Tau is essential to beta-amyloid-induced neurotoxicity. Proceedings of the National Academy of Sciences (PNAS) 99(9):6364- 6369, 2002

Rinne J, Koulu M: Alzheimerin taudin lääkkeet. In a book: Farmakologia ja toksikologia. 7.edition, p. 481- 488. Edited by Koulu M, Tuomisto J, Kustannusosakeyhtiö Medicina, Kuopio 2007

Rogers J, Strohmeyer R, Kovelowski CJ, Li R: Microglia and inflammatory mechanisms in the clearance of amyloid β peptide. Glia 40(2):260– 269, 2002

Rosenberg PB: Clinical aspects of inflammation in Alzheimer's disease. International Review of Psychiatry 17(6): 503– 514, 2005

Sarbassov DD, Guertin DA, Ali SM, Sabatinin DM: Phosphorylation and regulation of Akt/PKB by the Rictor- mTOR complex. Science 307(5712):1098- 1101, 2005

Simpson RJ, Adams PD, Golemis EA: Basic methods in protein purification and analysis. Cold Spring Harbor Laboratory Press, New York USA, 2009, pp.267- 268

Selkoe DJ: Translating cell biology into therapeutic advances in Alzheimer's disease. Nature 399(6738): A23- 31, 1999

Selkoe DJ: Resolving controversies on the path to Alzheimer's therapeutics. Nature medicine 17(9):1060- 1065, 2011

Shumaker SA, Legault C, Rapp SA, et al.: Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. JAMA 289(20):2651-2662, 2003

Shumaker SA, Legault C, Kuller L, et al.: Conjugated equine estrogens and incidence of probable dementia and mild cognitive impairment in postmenopausal women: Women's Health Initiative Memory Study. JAMA 291(24):2947- 2958, 2004

Tanzi RE, Bertram L: Twenty Years of the Alzheimer’s Disease Amyloid Hypothesis: A Genetic Perspective. Cell 120:545- 555, 2005

Rokhlin OW, Guseva NV, Taghiyev AF, Glover RA, Cohen MB: Multiple effects of N-alpha-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) on apoptotic pathways in human prostatic carcinoma cell lines. Cancer biology & therapy 3(8):761-768, 2004

Tokutake T, Kasuga K, Yajima R, Sekine Y, Tezuka T, Nishizawa M, Ikeuchi T: Hyperphosphorylation of tau induced by naturally secreted amyloid-β at nanomolar concentrations is modulated by insulin-dependent Akt-GSK3β signaling pathway. The journal of biological chemistry 287(42):35222- 35233, 2012

Vasudevan KM, Garraway LA: AKT signaling in Physiology and Disease. Current Topics in Microbiology and Immunology 347:105- 133, 2010

Venneti S, Wiley CA, Kofler J: Imaging microglial activation during neuroinflammation and Alzheimer's disease. Journal of neuroimmune pharmacology 4(2):227- 43, 2009

Vivanco I, Sawyers CL: The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nature Reviews Cancer 2:489- 501, 2002

Wharton W, Baker LD, Gleason CE, et al.: Short-term hormone therapy with transdermal estradiol improves cognition for postmenopausal women with Alzheimer's disease: results of a randomized controlled trial. Journal of Alzheimer’s disease 26(3):495- 505, 2011

Wiza C, Nascimento EBM, Ouwens DM: Role of PRAS40 in Akt and mTOR signaling in health and disease. American journal of physiology. Endocrinology and metabolism 302(12):1453- 1460, 2012

Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ: Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell 80(2):285- 291, 1995

Yeoman AD, Dew MJ, Rajapaksa S: Role of cognitive function in assessing informed consent for endoscopy. Postgraduate medical journal 82(963):65- 69, 2006

Yiannopoulou KG, Papageorgiou SG: Current and future treatments for Alzheimer's disease. Therapeutic advances in neurological disorders 6(1):19- 33, 2013

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ: Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 87(4):619- 628, 1996

Zhao J, Meyerkord CL, Du Y, Khuri FR, Fu H: 14-3-3 proteins as potential therapeutic targets. Seminars in cell & developmental biology 22(7):705-712, 2011

Zhao W-Q, Townsend M: Insulin resistance and amyloidogenesis as common molecular foundation for type 2 diabetes and Alzheimer's disease. Biochimica et biophysica acta 1792(5):482- 496, 2009

Zurashvili T, Cordón-Barris L, Ruiz-Babot G et al: Interaction of PDK1 with phosphoinositides is essential for neuronal differentiation but dispensable for neuronal survival. Molecular and cellular biology 33(5):1027-1040, 2013